GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF RESEARCH ADMINISTRATION

RESEARCH PROJECT INITIATION

Date: August 25, 1975

Project Title: Development of a Polygenic Assay for Induced Point Mutations in Mice

Project No: G-32-621

Principal Investigator Dr. John W. Crenshaw, Jr.

Sponsor: DHEW/PHS/NIH - National Institute of Environmental Health Sciences

Agreement Period: From June 25, 1975 Until June 24, 1979*

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GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION

Date: 6/22/81

Project Title: Development of a Polygenic Assay for Induced Point Mutations in Mice

Project No: G-32-621

Project Director: Dr. J. W. Crenshaw, Jr.

Sponsor: DHEW/PHS/NIH

Effective Termination Date: 8/25/79

Clearance of Accounting Charges: 8/25/79 (perf.)
9/25/79 (rpts.)

Grant/Contract Closeout Actions Remaining:

- x Final Invoice and Closing Documents
- _ Final Fiscal Report
- x Final Report of Inventions
- x Govt. Property Inventory & Related Certificate
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- _ Other _____

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G-32-621

REPORT OF PROGRESS FOR RESEARCH CONTRACT NIEHS NO. NO1-ES-5-2135

June 25, 1975 through December 24, 1975

The experiment proposed for the first year involved the determination of effect of a single mutagenic agent, administered in three different doses in addition to a negative control, upon three different germ cell stages, as reflected by eight phenotypic characteristics in an F_1 and an F_2 generation of mice of inbred strain DBA/2J. In addition to these characteristics, and for purposes of comparison with the results of other mutagenic studies, a dominant lethal test was planned for parental generation females mated with mutagenized (or control) parental males. The mutagenic agent selected for use in the first experiment was triethylene melamine (TEM), and dosages selected for administration were .075, .15, .30 milligrams per kilogram of body weight, in addition to an injection of carrier only to control males. The phenotypic traits selected for study included (1) number of young per female carried to weaning, (2) age of eruption of incisors, (3) age of development of the righting response, (4) mean and variance of body weight at weaning, (5) length of tail at seven weeks of age, (6) sex ratio, (7) hematocrit at seven weeks, and (8) serum cholestrol level at seven weeks.

In general, the experiments have proceeded satisfactorily except for minor problems relating to delayed arrival of certain items of equipment and supplies and to a limited loss of mothers and litters, presumably due to disease, probably of a bacterial nature. The numbers of F_1 generation young available for the establishment of F_1 crosses for all three mutagenized germ cell stages are adequate to provide useful information. The first F_1 crosses are scheduled to be established shortly after the beginning of the second half year, in late December, 1975.

Design. Parental females serving as parents for determination of the effects of different germ cell stages were termed groups A, B and C to indicate respectively those inseminated by spermatazoa which had been exposed to mutagenic action as spermatozoa, spermatocytes and spermatogonia. According to the original plan a total of 144 male mice would be treated at ten weeks of age and (1) would be mated immediately with group A females for a period of one week, then with females not involved in the experiment for a period of two weeks, at which point (2) the males would be mated with group B females for a period of two weeks followed by a period of two weeks again housed with females not involved in the experiment. (3) Matings would then be established between the same males and group C females, again for a period of two weeks. At this point, the parental generation males which had been mutagenized and those selected to serve as controls were to be destroyed. This constitutes in essence the portion of the experiment scheduled for completion within the first half year.

Methods. Parental male and group A female mice were received from the Jackson Laboratory at eight weeks of age on September 16, 1975, as ordered. Both males and females were housed five to a box, the females for a period of two weeks until initial matings were established. Males were housed separately for the second week, immediately prior to establishment of matings. Triethylene melamine was administered by intraperitoneal injections in Hank's Balanced Salt Solution prepared immediately prior to injection. A series of male mice were weighed to determine average body weight, and all males within a dose group received the same quantity of carrier and TEM irrespective of individual variation in body weight. All mice received .25 cc of solution by injection, controls receiving the same quantity of carrier only.

Injections were carried out on September 30, 1975, and matings were established, three females to each male, immediately after injection. All males treated survived for a period of 24 hours, and, in fact, until all were destroyed at the completion of the portion of the experiment involving them.

For purposes of recognition, Group A females were numbered from 1 to 436 and were ear punched according to a numerical identification code. Males were similarly ear punched for identification and were numbered 501 through 645. Males were randomly assigned to one of the three experimental or control groups, each group thus containing 36 males. In similar manner, females were assigned randomly to males within the four different groups. Following establishment of group A crosses, cages were labeled in such a way as to preclude group identification.

Group A matings were established on September 30, 1975; males were removed on October 7, 1975, and placed with females not involved in the experiment in an effort to maintain a normal level of sexual behavioral activity in the males. Ten days later, in expectation of production of litters, trios of mated females were separated one ot a box.

Group B females were received from the Jackson Laboratory on October 8, 1975, at eight weeks of age, 427 in number (5 fewer than had been ordered). These females were housed five to a box for the twelve days prior to the establishment of matings on October 20, 1975; females were ear punched to reflect numerical identification from 701 to 1130. As in group A matings, three females, randomly selected, were housed with each male until the males were removed on November 3, 1975.

Unfortunately, delay in the arrival of double-compartment polycarbonate boxes required that these matings be established in stainless steel mouse boxes. However, all four groups were similarly housed, and this was judged to be the best of the alternative solutions available.

Following removal from group B females on November 3, 1975, males were housed with females not involved in the experiment until the establishment of group C matings. The pressures of other demands of the project necessitated that the establishment of group C matings be handled on two successive days, November 19 and 20, 1975. Again, because of the delayed arrival of polycarbonate boxes, it was necessary to employ stainless steel boxes of the same type used for group B matings. The females involved in these experiments had been received from the Jackson Laboratory on November 5, 1975, at eight weeks of age, ear punched for numerical identification from 1201 to 1636, and housed, five to a box, until matings were established. An excess of five mice in this shipment permitted the establishment involving random selection of individuals, of the full series of 144 matings (one male: three females). Males were removed from the matings established on November 19 and 20, respectively on December 3 and 4, 1975, and destroyed. Subsequently, females were isolated, one to a box, in anticipation of production of litters.

The above summary deals with the establishment and maintenance of animals involved in the matings. The section below will deal with the handling and maintenance of animals of the F_1 litters produced.

F_1 young were assigned temporary numbers, within litter, at birth and this number was coded for identification by toe clippings. Subsequently, at weaning, a permanent number was assigned and the animals identified by ear punching.

For the Group A series a total of 187 litters were produced, based upon the observation of one or more young in a cage, between October 20, 1975 and October 27, 1975. A total of 126 of these litters had some young surviving at weaning. The distribution of litter size at birth and at weaning is shown in Table 1.

* * * * *

Table 1. Size distribution of Group A litters at birth and at weaning, including three experimental and one control group. Females producing these litters were mated for a period of seven days immediately following treatment.

litter size	1	2	3	4	5	6	7	8	9	10	Total
number of litters:											
at birth	6	32	44	35	28	22	10	8	1	1	187
at weaning	1	16	31	26	24	16	8	2	1	1	126

* * * * *

Of the 61 litters lost, 17, representing 95 young or about 41% of the total of 230 F_1 losses, were incurred during the (apparent) disease epidemic that was limited to mothers nursing relatively large litters. The mean litter size at birth (4.10) is similar to that at weaning (4.26) while the mean litter size lost as a result of mothers dying prior to weaning was 5.6. Analysis of the numbers of young remaining in each of the four groups indicates that all of the 17 litters lost were in either the control group (9 litters) or the group receiving the lowest TEM dose, .075 mg/kg body weight (8 litters).

In each of the four groups of Series A, control and experimental, more males survived to weaning than females. Accordingly, the number of females will probably determine the number of F_1 pair crosses that may be established. As it turns out, the number of females presently surviving to weaning in the control, .075 mg/kg, .15 mg/kg and .30 mg/kg groups are respectively 93, 82, 50 and 20. This suggests that we may expect to obtain relatively meaningful data for the F_2 generation for the control and .075 mg/kg body weight groups, perhaps something of interest from the .15 mg/kg body weight group, but little of significance from the .30 mg/kg body weight series. Nevertheless, we will establish as many crosses for each group as is possible for whatever information may be obtained.

Autopsy of group A parental females has been completed, and preliminary results of uterine examinations will be outlined below. For the group A F_1 young, data have been recorded for all with respect to sex, time of development of righting response, time of incisor eruption, and weight at weaning. We are currently in the process of obtaining data on tail length, hematocrit and cholesterol level. The analysis of these date will be presented in the second semi-annual report.

Group B series mothers produced a total of 309 litters, based upon the observation of one or more young in a box, between November 9 and November 24, 1975. This represents a great increase over the productivity of group A females, reflecting not

only the greater length of time the mated pairs were together but likely also a significant decrease in losses due to dominant lethality, in the progeny of mutagenized males. At weaning, 229 litters were still represented by surviving young. The distribution of litter sizes for this group at birth and at weaning is shown in Table 2.

* * * * *

Table 2. Size distribution of Group B litters at birth and at weaning, including three experimental and one control group. Females producing these litters were mated for a period of fourteen days beginning twenty days after administration of mutagenic agent, to the male parent.

litter size	1	2	3	4	5	6	7	8	9	10	Total
number of litters:											
at birth	16	27	45	62	55	45	30	26	1	2	309
at weaning	0	9	40	57	51	35	20	16	0	1	229

* * * * *

As was the case with group A series females a considerable part of the loss of young was due to the disease epidemic primarily affecting, again, mothers nursing relatively large litters. Of the total of 80 litters lost between birth and weaning 20, representing 142 young or 42% of the total of 336 F_1 losses, were lost due to this cause. The mean litter size at birth for all group B females, 4.68 was somewhat larger than for group A females but was, again, similar to their own mean litter size at weaning (4.84). As suggested above, the larger litter sizes probably reflect a reduction in dominant lethal losses, particularly among the two experimental groups receiving the highest mutagen doses. Mean litter size of diseased mothers dying prior to weaning (7.1) was much larger than that of surviving litters and somewhat larger than that of group A series females similarly lost. Unlike the situation in group A, the 20 litters lost were distributed over all four groups rather than being limited to females of the control and lowest mutagenic dose series.

Again, in each of the three experimental and one control groups, more males survived to weaning than females. Thus, the number of females will determine for the group B series the number of F_1 pair crosses that may be established. At the present time, the number of females surviving in the control, .075 mg/kg, .15 mg/kg, and .30 mg/kg groups are respectively 135, 142, 122 and 108. These numbers obviously will provide the basis for a respectable number of crosses for each group. These groups will be established in January, 1976.

The autopsy of group B parental females is being completed as this report is written, and time constraints prevent the presentation of these results. For these F_1 young, data have been recorded with respect to sex, time of development of righting response, time of incisor eruption, and weight at weaning. Analysis of these data as well as those on tail length, hematocrit and cholesterol level at seven weeks, will be presented in the second semi-annual report.

Group C series mothers are still producing litters as this report is being prepared and will be treated in the next semi-annual report. An apparent disease epidemic similar to that in other groups is in progress, but numbers will probably be comparable to those in Group B.

Results. A summary of the data on dominant lethality and related losses in Group A series females is given in Table 3.

* * * * *

Table 3. Dominant lethality parameters of Group A series females and their F_1 young. Females belonging to the three experimental and single negative control group were mated for a period of seven days immediately following treatment.

	Dose of TEM in mg/kg of body weight			
	0	.075	.150	.300
% infertile ♀♀ of total mated (sample)	32.4(108)	27.4(106)	34.9(106)	57.4(108)
% "dead" scars of total (sample)	10.7(67)	18.6(73)**	21.3(69)**	20.4(46)**
total scars ("live"+ "dead") per fertile ♀(sample)	6.27(67)	5.88(73)	5.10(69)	4.37(46)
live births based upon "live" scars, if known, or observed young per mated ♀ (sample)	3.82(73)	3.49(77)	2.61(69)	1.47(46)
% losses at birth (= "live" scars - observed live births)	32.3(67)	30.7(73)	46.2(69)	56.0(46)

** $P < .005$ in comparisons with control

* * * * *

The statistical results provided are based upon X^2 contingency comparisons, and definitive conclusions must await more critical statistical evaluation. However, the primary value of these data is that it is clear that effective administration of mutagenic agent has been effected. Further, there are several indications of increased response to increasing dose. Live births per mated female show a clear trend. It should be borne in mind that figures for the two highest doses are based entirely upon "live" scar counts. "Observed young per mated female" are included only in figures for the control and lowest dose groups in which some mothers were lost to disease prior to autopsy. The interpretation of increasing post-partum losses with increasing dose is difficult for, as pointed out by Soares, (1972, Jour. Heredity 63(6):339), it is possible that there may be involved both the expression of dominant lethality at birth and the increased loss of young associated with small litters and, presumably, inadequate maternal care.

Submitted by: John W. Crenshaw

John W. Crenshaw, Jr.
Project Director

REPORT OF PROGRESS FOR RESEARCH CONTRACT NIEHS NO. NO1-ES-5-2135 6-32-621

December 25, 1975 through June 22, 1976

The experiment proposed for the first year involved the determination of effect of a single mutagenic agent, administered in three different doses in addition to a negative control, upon three different male germ cell stages, as reflected by eight phenotypic characteristics in an F_1 and an F_2 generation of mice of inbred strain DBA/2J. In addition to these characteristics, and for purposes of comparison with the results of other mutagenic studies, a dominant lethal test was planned for parental generation females mated with mutagenized (or control) parental males. The mutagenic agent selected for use in the first experiment was triethylene melamine (TEM), and dosages selected for administration were .075, .15, .30 milligrams per kilogram of body weight, in addition to an injection of carrier only to control males. The phenotypic traits selected for study included (1) number of young per female carried to weaning, (2) age of eruption of incisors, (3) age of development of the righting response, (4) mean and variance of body weight at weaning, (5) length of tail at seven weeks of age, (6) sex ratio, (7) hematocrit at seven weeks, and (8) serum cholesterol level at seven weeks.

The experiments planned for the first year have been completed satisfactorily at this point except for the spectrophotometric analysis of cholesterol levels in plasma samples and for some specialized statistical analyses of data. Cholesterol determinations have required a greater time investment than anticipated. Of course, judgement as to whether this characteristic is worth the time investment required to employ it must await the final analysis of these data which should be completed during the summer. For all other traits it is felt that the number of young of both the F_1 and F_2 generations are satisfactory to provide at least the pilot indications to indicate the potential usefulness of the traits employed.

Design. Parental females serving as parents for determination of the effects of different germ cell stages were termed groups A, B. and C to indicate respectively those inseminated by spermatozoa which had been exposed to mutagenic action as spermatozoa, spermatocytes and spermatogonia. According to the original plan a total of 144 male mice would be treated at ten weeks of age and (1) would be mated immediately with group A females for a period of one week, then with females not involved in the experiment for a period of two weeks, at which point (2) the males would be mated with group B females for a period of two weeks followed by a period of two weeks again housed with females not involved in the experiment. (3) Matings would then be established between the same males and group C females, again for a period of two weeks. At this point, the parental generation males were to be destroyed.

For all groups, females were isolated from males and other females by 18 days after initiation of matings so as to prevent postpartum matings and to avoid confusion of assignment of young to a particular litter, or a particular litter to a female. As detailed in the first semi-annual report, young were marked for identification and the sex recorded at birth. Subsequently, data on the time of development of the righting response and eruption of upper incisors were recorded. Approximately 22 days after males had been removed from contact with females in a given group, autopsy of unproductive females was initiated.

Young were weaned at four weeks of age. At weaning the young were permanently marked by ear punching, and the sex and body weight were recorded. At approximately seven weeks of age, tail length was recorded and a blood sample was obtained, hematocrit level recorded and the plasma frozen and stored for cholesterol level determination at a later date.

F_1 matings were established with individuals between 11 and 12 weeks of age, but full sibling pairs were precluded. F_1 pairs remained together for a period of two weeks at which time males were removed and destroyed. The data acquired for F_2 litters were the same and were taken at the same time intervals as for F_1 young except that F_1 females were not autopsied for intrauterine scar data. Unproductive females were destroyed about 21 days following the removal of males, and productive females were destroyed after their litters had been lost or weaned.

Methods. The procedures involved in receiving mice, in mutagen administration, maintaining mice and establishing crosses, handling young, and acquiring data on traits were detailed in the report of progress for the first half year. Summaries were provided of number of litters produced, surviving at weaning and distribution of litter size at birth and at weaning for groups A and B. A summary of these data for the remaining series follows.

Group C series mothers produced a total of 269 litters, based upon the observation of one or more young in a box between December 6 and December 24, 1975. This is more comparable to the productivity of B series mothers (309 litters) than it is to the productivity of A series mothers (187 litters). It is likely that this reflects not only the greater length of time the mated pairs were together in both Groups B and C (two weeks) than in group A (one week), but, more importantly, there were significant decreases in losses due to dominant lethality in Groups B and C in the progeny of mutagenized males. At weaning, 192 litters were still represented by surviving young in Group C as compared with 126 in Group A and 229 in Group B. The distribution of litter sizes for Group C at birth and at weaning is shown in Table 1.

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Table 1. Size distribution of Group C litters at birth and at weaning, including three experimental and one control group. Females producing these litters were mated for a period of 14 days beginning 52 days after administration of mutagenic agent to the male parent.

litter size	1	2	3	4	5	6	7	8	Total
number of litters:									
at birth	9	35	35	49	44	42	38	17	269
at weaning	3	7	28	41	49	27	29	8	192

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As was the case in both Group A and Group B series, part of the loss of young was due to a disease epidemic primarily affecting mothers nursing relatively large litters. Of the total of 77 litters lost between birth and weaning six,

representing 44 young or 14 per cent of the total of 315 F_1 losses in this group, were lost for this reason. Percentage losses in Groups A and B were about three times as large as those of Group C, because productive Group C females were automatically treated with antibiotic during the regularly scheduled box changes on the week following litter production. The mean litter sizes at birth and at weaning are compared in Table 2.

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Table 2. Overall mean litter size of Groups A, B and C at birth and at weaning.

	mean litter size		
	Group A	Group B	Group C
At birth	4.10	4.68	4.66
At weaning	4.26	4.84	4.89

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As is indicated in Table 2, litter sizes of Group B and Group C females were quite comparable and are larger than those of Group A both at birth and at weaning. As suggested earlier, the larger litter sizes of Groups B and C probably reflect a reduction in dominant lethal losses, particularly among the two experimental groups receiving the highest mutagen doses. Mean litter sizes of diseased mothers of Group C dying prior to weaning (7.3) was much larger than that of surviving litters, slightly larger than that of Group B females (7.1) and considerably larger than that of Group A series females (5.6) similarly lost.

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Table 3. Dominant lethality parameters with standard errors and sample sizes of Group A series females and their F_1 young. Females belonging to the three experimental and a single negative control group were mated for a period of seven days immediately following treatment, thus represent effects of mutagenic agent upon spermatozoa. (Live scar data were not tested statistically).

	TEM dose, mg./kg. of body weight			
	0	.075	.15	.30
Live Scars/♀	3.63 ± .37(98)	3.32 ± .28(97)	2.59 ± .27(107)	1.47 ± .22(108)
Dead Scars/♀**	.42 ± .07(98)	.78 ± .11(99)	.70 ± .10(107)	.39 ± .08(108)
Total Scars/♀*	4.01 ± .36(98)	4.10 ± .32(99)	3.29 ± .30(107)	1.86 ± .25(108)
Total Live Births/♀**	4.50 ± .27(52)	4.17 ± .22(54)	3.47 ± .27(43)	3.18 ± .29(22)
Total Weaned/♀*	4.97 ± .27(39)	4.20 ± .25(41)	3.86 ± .32(28)	3.74 ± .36(14)

* Difference among groups, $P < .05$
** Difference among groups, $P < .01$

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Results. Analyses of variance of data on dominant lethality and related losses confirm the significant nature of the tentative results summarized in the Report of Progress for the first half year on Group A series females and their F_1 young.

Table 3 summarizes these results. Data on dead scars, presented in the Table as mean scars per female were analyzed as percentage of total scars after arc sine transformation. Total scars were analyzed after square root transformation. Differences among the control and three experimental groups were found to be significant for number of dead uterine scars per mated female, for total scars per mated female, for total number born per female bearing live young, and for total number weaned per female carrying young to weaning. The trends are in the direction expected with live scars, total born, and total weaned per female varying inversely with mutagenic dose. Total scars varied in a similar manner except that there was a nonsignificant increase in total scars from the control to the lowest mutagenic dose followed by a significant linear decrease in number of scars in the higher dose rates. The reduction in total scar numbers for the two higher doses are interpreted as resulting from preimplantation losses which would be expected to increase at high mutagenic agent exposures. As expected from earlier studies total number of dead scars increased from control to the two lower mutagenic doses but dropped off in the highest dose to a level comparable with controls. The hypothetical relationship between live scars, dead scars and preimplantation losses in Group A series females is shown in Figure 1.

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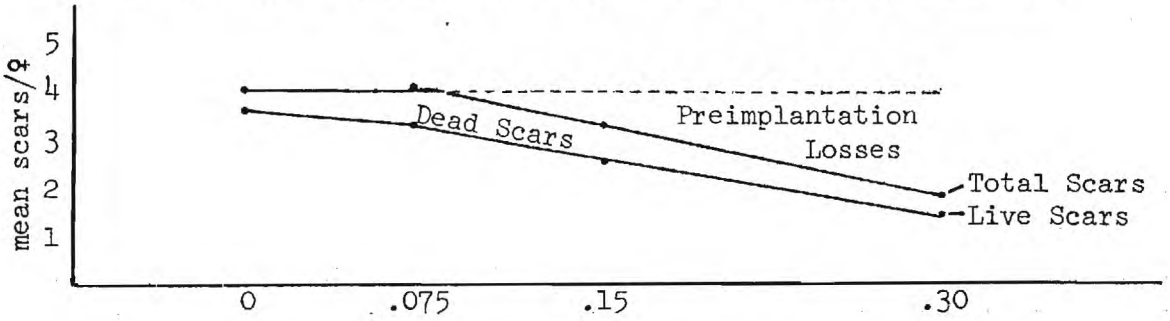
Figure 1. Relationship between per female live scars, dead scars and estimated preimplantation losses for females of three experimental and a single negative control group showing the effects of mutagenic agent upon spermatozoa.



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The simplest interpretation of the data from Group A females is that at the lowest dose of TEM, .075 mg./kg. of body weight, no significant preimplantation losses occur. However, at this dose there is an apparent although not significant increase in the number of dead scars precisely compensated for by a reduction in the number of live scars per female. On the assumption that the reduction in total scars at the higher TEM doses, .15 and .30 mg./kg., is due to preimplantation losses, the total number of losses due to mutagenic agent (the sum of preimplantation losses and postimplantation losses represented by dead scars) increases with a proportionate decrease in the total number of live scars per female at higher doses. This is consistent with the results of earlier studies by Matter and Generoso (1974).

In Figure 2 are shown the dose response curves developed by Matter and Generoso (ibid) with data from the present study superimposed for comparison. These

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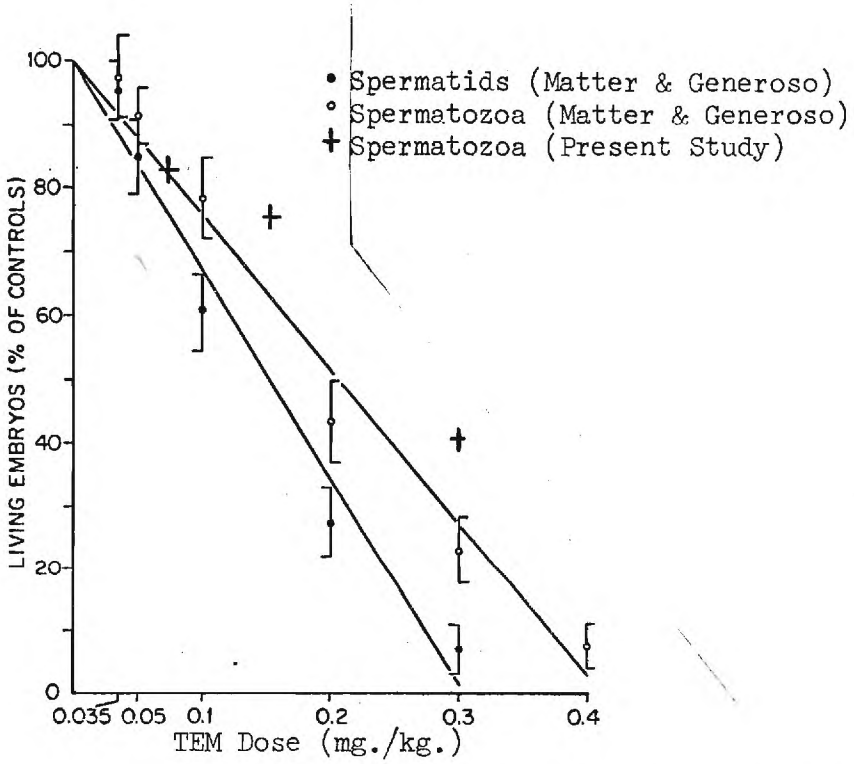

Figure 2. Effect of TEM dose on induction of dominant-lethal mutations in mouse spermatozoa and spermatids. The curves represent least-squares fits of the numbers of living embryos vs. the doses. Ninety-five percent confidence intervals are indicated by vertical bars. Taken from Matter and Generoso (1974) with results of the present study added for comparison.

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curves are based upon living embryos as a percent of living embryos in the control group. So that our observations could be compared directly with theirs, we have calculated, as they did, live scars per mated female for the .3 mg./kg. group as a percent of controls, but for lower doses we have employed live scars among fertile females only as a percent of controls. Given the differences that exist between our method and theirs (i.e., our use of an inbred strain as opposed to an interstrain hybrid, and postpartum analysis of uterine scars in our study as opposed to uterine analysis at 12 to 15 days following plugging in the Matter-Generoso study) our results are quite comparable. The slope of our curve would appear to result in a slightly lesser rate of reduction in living embryos with increasing dose than the Matter and Generoso curved.

It was observed by Soares (1972) that the number of live scars observed was virtually always either equal to or more than the number of live young observed at birth. Soares interpreted this difference as being due either to a late dominant lethal effect or to an increased loss of young associated with small litter sizes which would be more common at higher mutagen doses. We are carrying out an analysis of our data on this phenomenon based upon regression of number of losses-at-birth upon litter size for the control and treated series involved in our study. From these data it should be possible to determine the effect of litter size upon losses at birth.

For females of the Group B series, the young of which developed from ova fertilized by spermatozoa subject to mutagenic treatment as spermatocytes, there were slight, though statistically significant evidences of an effect. These results are shown in Table 4. Significant variation was found among the three experimental and single control group in regard to total scars per female. These

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Table 4. Dominant lethal parameters with standard errors and sample sizes of Group B series females and their F_1 young. Females belonging to the three experimental and single negative control group were mated for a period of 14 days beginning three weeks after treatment, thus representing effects of the mutagenic agent upon spermatocytes.

	TEM dose, mg./kg. of body weight (sample size)			
	0(98)	.075(100)	.15(95)	.30(98)
Live Scars/♀	4.76 ± .29	5.60 ± .24	4.81 ± .28	4.46 ± .25
Dead Scars/♀*	.58 ± .09	.66 ± .08	.81 ± .10	.73 ± .10
Total Scars/♀**	5.34 ± .31	6.26 ± .24	5.62 ± .31	5.19 ± .28

*p = .052
**p < .05

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conclusions are based upon an analysis of variance of number of total scars per female after square root transformation. Data on dead scars were analyzed by ANOVA after arc sine transformation of the dead scar percentage of total scars. Dead scars per female increased above control with increasing dose to fall slightly at the highest dose. This pattern would not be particularly unusual if there were reason to suppost that preimplantation losses had resulted in a reduction in total implantations at the highest dose. However, total scars increased from control to a peak at the lowest TEM dose from which they decreased with further increase in dose so that total number of scars in the highest TEM dose is slightly lower than that of the control. A multiple range test indicated that the lowest TEM dose was associated with a significantly higher number of total scars than either the control or the highest TEM dose, and was responsible for the significant variation among the four groups.

In the study by Matter and Generoso, no effects of mutagenic treatment on

spermatocytes were reported below doses of .8 mg./kg. However, at .4 mg./kg. there were observed non-significant increases above controls in total implants in zygotes fertilized by spermatozoa mutagenized as late spermatocytes. This may indicate an interesting if paradoxical effect of a low dose TEM treatment causing an increase in total implants.

It is difficult to account for the kind of variation observed in our study on any straightforward biological basis. If real, increased implants could be due to mobility or physiological activity. It is, of course, possible that the peak value in total scars for the lowest dose, .075 mg./kg. of body weight, represents chance variation of a relatively infrequent sort, and has led us to commit a Type I error. Certainly there are no other evidences in the spermatocyte treated series (Group B), either in the F_1 or the F_2 generation, to indicate that mutations have been induced by treatment at any dose level.

With respect to dominant lethal measures for Group C series females, those producing young which were the products of fertilization by a spermatozoan exposed to mutagenic treatment in the spermatogonial stage, there were no significant differences observed among groups for any dominant lethal measure. For this group, however, there are other evidences that mutations were induced (see discussion of quantitative effects on spermatogonia, page 14). It seems likely that the kinds of mutations induced were limited to lesions with minor effects on fitness traits.

Quantitative Traits. While the data above on dominant lethal measurements provide some confirmation of results by earlier workers (Matter and Generoso, 1974) and some new information, the primary thrust of the present study was directed toward the utilization of quantitative traits as indicators of mutagenic activity. Earlier studies have indicated that it is desirable to look not only at the effects of polygenic mutations, that is mutations of minor effect, on the means of quantitative traits but also their effect upon the variances of such traits (Crenshaw, 1970; Soares and Crenshaw, 1970). While information from either the F_1 or F_2 generation may be of interest in pointing out a deleterious effect resulting from treatment with a particular compound, in general it has been our assumption that effects observed in the F_2 generation were more surely attributable to a genetic basis than those of the F_1 generation. However, genetic cause obviously cannot be excluded from F_1 effects.

Effect of Mutagen on Spermatozoa

In young of the F_1 generation significant effects of mutagen dose were observed with respect to means for body weight, tail length and hematocrit. With respect to body weight an analysis of variance indicated that there were highly significant differences among doses and between sexes. The differences due to dose became even more impressive when litter size was considered ($F = 4.13$, $P < .01$). No evidence of a dose x sex interaction was found.

It is evident from Table 5 that mean body weight of F_1 progeny increases directly with dose. The results of a multiple range test show that the control is significantly different from both the low and the middle TEM dose, but that the three experimental groups do not differ significantly from one another. While the

highest group (.30 mg./kg.) mean is the highest of the series in face value,

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Table 5. Means of body weight at weaning, and tail length and hematocrit levels at seven weeks in F_1 and F_2 generation progeny of three experimental groups and a single negative control showing effects of TEM upon spermatozoa. Sexes are combined. Means over solid lines at the same level, whether continuous or connected by dashed line, are not significantly different by multiple range comparison.

	F_1				F_2			
Dose (mg./kg.)	0	.075	.15	.30	0	.075	.15	.30
Body weight	13.06	13.54	13.94	14.05	12.79	13.19	10.87	13.00
Tail Length	69.75	70.82	70.80	70.58	71.40	71.90	68.60	71.33
Hematocrit	48.24	48.42	48.67	48.79				

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failure to attain significance of the difference from control was due to small sample size. The treatment effect of increasing body weight is unique among similar studies of the effects of (other) mutagenic agents upon body weight. In other studies, body weight of the F_1 young has been found to be reduced in some studies, and in some there has been no effect on mean. It is our feeling that the different kinds of responses which the particular experimental design employed in these studies makes possible, provides potential for differentiating between the effects of different types of mutagenic agents. It is hoped that the importance of this kind of variation in response may be developed more fully in our experiment projected for a future year, which will compare the effects of different mutagenic agents.

An analysis of the effects of sex and litter size on body weight reveals that the pattern of linear increase of body weight with increasing dose is characteristic of males, and that the variation in pattern among different litter sizes, particularly in the more numerous litter size categories, is negligible. Females of the F_1 generation show a slightly different pattern, involving a modest decrease in body weight from the middle to the highest TEM dose. However, this decrease is certainly not significant and seems to be characteristic of smaller litter sizes which are sufficiently numerous at the highest dose rate to have an appreciable effect on the mean.

Mean tail length follows a similar pattern to that of body weight, with significant differences among the dose groups ($F = 3.99$, $P < .01$). While tail length does not appear to increase directly with increasing dose as was the case with body weight, the means for all experimental groups of the F_1 generation are greater than those of the control group. Inasmuch as tail length is a measurement that may be obtained with greater facility than body weight, some small economy might be

achieved by employing this measurement in lieu of body weight. However, as will be discussed shortly, there are problems with the use of tail length variance that are not present in the body weight measurement.

A comparison of the effect of sex and litter size upon tail length indicates that the experimental groups, both males and females, exhibit greater tail lengths than the control group. There is, however, considerable variation among litter sizes as well as among doses so that general patterns do not emerge. Most litter sizes, for both males and females, do show an increase in tail length from the control to the lowest TEM dose.

Hematocrits of the F_1 progeny follow the same pattern as those for body weight and tail length and, in fact, like body weight do appear to increase directly with increasing dose. $P < .05$ ($F = 2.62$) that the differences observed between groups could be due to chance alone. Only the middle TEM dose group is significantly different from the control, but the high dose group mean suffers from small sample size.

In an analysis of the effect of sex and litter size on variation in hematocrit level, the patterns for males and females were found to be quite similar with the exception that in males all experimental groups show higher hematocrit levels than controls, while in females only the two highest TEM dose groups show hematocrit levels above the control group, with the lowest TEM dose group having hematocrit levels very slightly below that of controls. Relative to variation in body weight and tail length among litter size groups, that in hematocrit level is very limited.

Examination of the effect of sex and litter size upon body weight in the F_2 generation young reveals significant differences among dose groups ($P < .001$, $F = 14.57$) considering both sex and litter size. Significant reduction in body weight at the middle TEM dose (.15 mg./kg.) is responsible for this result and is particularly remarkable because it reflects a reduction in the mean body weight of individuals categorized by sex and litter size in the middle TEM dose, as compared to all other dose groups. The significant increase in body weight from the middle to the highest TEM dose, while it is significant, does not reflect as consistent a pattern among litters as the decrease from lowest to middle TEM dose group. Repetition of this particular experiment in the coming year will be of great value as a test of the reality of the effect of induced variation upon the body weight of F_2 progeny.

In earlier studies of the effects of mutagenic agents on body weight in the F_2 generation, the usual result has been no effect on mean. In a few instances, reductions in weight similar to that discussed above, have been reported. The present result suggests that dose may be critical in producing the precise quality and amount of damage that will lead to a reduction in body weight.

As was the case with the F_1 generation results, results of tail length comparisons among litter sizes and between sexes parallels very closely with variation patterns in body weight. An analysis of variances of differences among dose groups, considering both covariance with litter size and differences due to

sex, reveal that the differences are significant ($P < .05$, $F = 3.22$). As shown in Table 5, there is a significant reduction in tail length, precisely paralleling the result with body weight, in the middle TEM dose group. In general both males and females exhibit similar patterns, with mean tail length for the low TEM dose in both males and females being very slightly greater than controls. For the high dose group, females exhibit slightly greater tail length than controls but males have slightly lesser tail lengths. Both males and females are characterized by a fairly consistent reduction in tail length for the middle TEM dose, with three of five of the litter sizes including females and four of five of the litter sizes including males exhibiting parallel tail length reductions from the low to the middle TEM dose. At the highest dose level there is remarkably little variation among litters in respect to tail length means. However, considering the small sample sizes involved, this may be a chance effect.

Turning to a consideration of variances of the same traits considered above (body weight, tail length and hematocrit level) certain patterns emerge that are of interest. Table 6 presents in tabular form variances for male

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Table 6. Variances of body weight at weaning, and tail length and hematocrit levels at seven weeks in F_1 and F_2 generation progeny of three experimental and a single negative control groups showing effects of TEM upon spermatozoa. Indications of statistical significance pertain to comparisons with appropriate controls and are based upon variance ratios.

		F_1				F_2			
Dose (mg./kg.)		0	.075	.15	.30	0	.075	.15	.30
Body Weight	♂	7.62	4.45*	6.81	2.46**	5.95	8.01	8.58	14.29*
	♀	5.34	3.50*	4.28	2.02**	7.24	7.90	11.56	8.41
Tail Length	♂	9.67	9.99	12.18	4.04**	4.84	9.12**	9.86	14.98**
	♀	13.03	9.80	9.24	6.71	6.92	9.61	46.51**	9.55
Hematocrit	♂	1.74	1.64	3.20**	1.56	1.32	2.56**	1.85	1.61
	♀	2.10	2.37	3.80*	2.16	1.96	2.34	4.75*	1.90

* $P < .05$
** $P < .01$

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and female groups in the F_1 and F_2 generation for the negative control and three TEM dose groups involved in our study. One of the hypotheses that has emerged from earlier studies of polygenic mutations is that there appears to be a characteristic decrease in variance with respect to body weight in F_1 generation young of a mutagenized male parent. Our own results from the present study provide

strong confirmation of this effect. For all three TEM doses for both male and female samples there is a reduction in body weight variance among the progeny of treated males, and four of the six comparisons possible produce significant differences based upon F ratio comparisons.

Tail length among females showed a similar pattern of reduced variance of the experimental groups as compared with the control group but only one comparison was significant. However, males exhibited a different pattern. In both the lower and middle TEM dose groups there were non-significant increases in variances over controls. Males of the highest TEM dose group did exhibit a highly significant reduction in variance relative to that of controls.

With respect to variance differences among groups in regard to hematocrit level, the same group that exhibited a significant increase in mean hematocrit level, the 15 mg./kg. dose group, also exhibited a significantly increased variance with respect to hematocrit level for both sexes, which may be due in part to the direct relationship between mean and variance.

Previous studies in our laboratory have developed evidence that, just as body weight variance is often decreased relative to control groups in the F_1 progeny of mutagenized males, so body weight variance is often increased in the F_2 generation progeny of offspring of mutagenized males. The prediction leading from this hypothesis is strongly confirmed in the results of the present study, not only in regard to body weight variances but also by tail length variances and in all except one of the possible hematocrit variance comparisons. To be precise, the variances in regard to both body weight and tail length in F_2 generation young of all three TEM dose groups for both sexes (a total of 12 comparisons) are higher than the appropriate control variance. Only one-third of these variance ratio comparisons produce significant F values, however it is evident that the likelihood of having all twelve comparisons fall in the same direction by chance along is remote (P is approximately equal to .00024). In the hematocrit variance comparisons, all except one of the six experimental group variances is higher than the appropriate control, and two of these are significantly so. The effects of mutagenic treatment on the F_1 and particularly the F_2 generation on the variance of quantitative traits support the hypotheses of earlier studies.

In the discussion above of the effect of the mutagenic agent TEM upon spermatozoa, we have limited the treatment to statistically significant results. There are interesting implication in some of the other aspects of our data, for example with respect to sex ratio, the time of development of the righting response and the time of emergence of upper incisors. Our analyses of these data are continuing and, if results of interest do develop, these will be reported in the next semi-annual report.

Effect of Mutagen on Spermatocytes

There are no strongly persuasive evidence in either F_1 or F_2 generation data that indicate an effect from mutagen induced polygenic mutations. In a single trait, tail length, there were highly significant differences in means due to dose when the effects of both litter size and sex were partitioned out (Table 7). The fact that such differences were observed for both the F_1 and F_2

generations lends strength to the likelihood that they are real, even in the absence of differences in means of other traits involved in the study.

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Table 7. Means of tail length at seven weeks, corrected for sex and litter size, in F_1 and F_2 generation progeny of three experimental groups and a single negative control showing effects of TEM upon spermatocytes. Sexes are combined. Means over solid lines at the same level, whether continuous or connected by dashed line, are not significantly different by multiple range comparison.

	F_1				F_2			
Dose (mg./kg.)	0	.075	.15	.30	0	.075	.15	.30
Tail Length	70.91	70.24	71.37	71.02	68.26	68.54	67.26	68.60

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In the F_1 generation it is in the lowest mutagen dose group that tail lengths were significantly different from (less than) those of the control group and, in fact, lower than the means for the two higher mutagen dose groups, although significantly different only from the middle dose. In the F_2 generation again one of the experimental groups exhibits a lower mean than that of the control and the other experimental groups, but in this case it is the middle TEM dose (.15 mg./kg.) that shows the lowest tail length mean.

It has been shown by Favor (1976) that the mean number of mutations in the F_1 and F_2 generations of progeny using the present design are not appreciably different. Accordingly, explanation for the kind of result observed here in respect to tail length is most simply attributable to maternal x progeny interaction. The F_1 generation progeny, which themselves may carry induced mutations, will be reared by a mother having no genetic damage. On the other hand, progeny of the F_2 generation will be reared by a mother that has approximately the same number of mutations as they themselves have. At this time, it would be somewhat speculative to attempt to account precisely for the mean differences observed here, but it would seem to be most worthwhile to seek answers in the differences between maternal effects of the two generations involved. It is worth noting that body weight means differences parallel very closely the differences found in tail lengths in both the F_1 and F_2 generations. These differences, however, do not approach significance when litter size and sex are considered.

With respect to differences in variance, only one pattern of interest was noted. With regard to time of incisor emergence, there was observed a consistent reduction in variance in the F_1 generation young of all three experimental groups relative to the control (Table 8). In two of the six comparisons possible, the differences were significant.

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Table 8. Variances associated with time of emergence of incisors in F_1 and F_2 generation progeny of three experimental and a single negative control groups showing effects of TEM upon spermatocytes. Indications of statistical significance pertain to comparisons with appropriate controls and are based upon variance ratios.

		F_1				F_2			
Dose (mg./kg.)		0	.075	.15	.30	0	.075	.15	.30
Incisor Emergence Time	♂	.7396	.6400	.6889	.6241	.9409	.7056*	.8281	.9801
	♀	.9025	.6400*	.7225	.5776**	.9801	.7744	.8649	.8100

* $P < .05$
** $P < .01$

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Variances for incisor emergence time are also shown for the F_2 generation in Table 8. The pattern suggested is the reverse of that when treated spermatozoa are involved, with evidences of a tendency for reduction of variance in experimental groups relative to that of controls in the F_2 generation just as there was in the F_1. The consistency of this directional difference is not complete, with one of the six comparisons possible showing an increase, albeit not significant, in the variance of incisor emergence time in an experimental group relative to that of controls. This apparent exception to the general trend is that for males in the high mutagen dose group. Only one of the other five differences proved to be significant by variance ratio comparison.

Since there were some indications of a mutagenic effect based upon dominant lethal parameters, it seems likely that the effects of dose on mean tail length and on variance of incisor emergence may be real. However, for practical purposes, the general insensitivity of spermatocytes to the mutagenic agent employed does not argue strongly for continued investigation of a treated spermatocyte stage in future experiments.

Effect of Mutagen on Spermatogonia

Even though there were no conclusive evidences of a dominant lethal effect in the F_1 young of males in which the spermatogonia had been exposed to the mutagen TEM, a number of evidences emerge from the quantitative traits analyzed to indicate that there were indeed polygenic mutations induced by TEM treatment, and that these were effective not only in modifying means but also variances of several traits, especially in the F_2 generation. Table 9 outlines the effects on trait means where differences among groups were found to be statistically significant.

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Table 9. Means of (1) time of development of righting response and (2) hematocrit level at seven weeks in the F_1 generation, and of (3) time of righting response, (4) time of incisor emergence, (5) body weight at weaning and (6) tail length at seven weeks in the F_2 generation progeny of three experimental groups and a single negative control showing effects of TEM upon spermatogonia. Means over solid lines at the same level, whether continuous or connected by dashed line, are not significantly different by multiple range comparison. Means have been corrected for sex and litter size.

	F_1				F_2			
Dose (mg./kg.)	0	.075	.15	.30	0	.075	.15	.30
Righting Response	7.67	7.93	7.74	7.98	8.37	8.30	8.08	8.18
Incisor Emergence Time					12.19	12.15	11.96	12.04
Body Weight					13.35	13.54	13.19	13.82
Tail Length					73.94	74.59	74.50	74.81
Hematocrit	47.39	47.13	46.80	47.36				

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With respect to the righting response, there were effects on the mean time of development of this behavioral trait due to treatment in both the F_1 ($F = 2.71$, $P < .05$) and F_2 ($F = 2.89$, $P < .05$) generations. In the F_1 generation the time of development of the righting response was delayed in all experimental groups relative to that of the control. However, the delay was significant only for the lowest and the highest TEM doses. In the F_2 generation, the effect appears to be reversed with the experimental groups all having shortened developmental times relative to the control. However, only the middle TEM dose is significantly different from the control, and it is not significantly different from the other experimental groups. The simplest interpretation of these data, assuming a negative correlation between fitness and developmental time, is that induced polygenic mutations showed a deleterious effect in F_1 generation progeny, but that reassortment of induced mutations, and perhaps selection against the more deleterious variants (and for any heterozygous

advantage) resulted in a reduced developmental time in the F_2 generation.

The only other trait to show a significant mean effect in the F_1 generation was hematocrit level ($F = 20.47$, $P < .01$), the general effect being one of reduced hematocrit in the three experimental groups. However, only in the case of the middle TEM dose was there a significant reduction, and this mean was significantly different from the control and the other experimental groups. Both sexes showed parallel reductions at the middle TEM dose, but males exhibited greater differences among means that females. Differences between sexes were reinforced by similar performances of different litter size groups within sex. Hematocrit means did not exhibit significant variation in the F_2 generation. However, differences among dose groups were sufficiently great so that repetition of the experiment with the same results would result in significant differences upon the combined factorial analysis of the two experimental data sets. The order of the computed hematocrit means was more or less linear, ranging from a low for the control group to a higher level for the lowest TEM dose and even higher values for the middle and highest TEM doses, which were themselves quite similar. It is difficult to interpret a given change in hematocrit level as "good" or "bad", but it is probably well to bear in mind that different traits might very well respond in different directions to induced genetic variance.

Of the traits showing an effect only in the F_2 generation the time of incisor emergence showed significant differences among groups ($F = 3.15$, $P < .05$). There was a shorter time to emergence for experimental groups than for the control, paralleling that found for development of the righting response. However, only the emergence time for the middle TEM dose group proved to be significantly different from control.

The body weight and tail length traits showed similar effects of treatment in the F_2 generation of the spermatogonial treated groups. For tail length, highly significant differences were found among groups ($F = 5.82$, $P < .01$), and all three experimental groups exhibited significantly greater tail lengths than were found for controls. Body weight also showed highly significant variation ($F = 5.29$, $P < .01$), but only the highest TEM dose was associated with a body weight mean significantly higher than that of controls. Only one of the other two experimental group means, that for the lowest TEM dose, was higher at face value than the control. Both body weight and tail length were characterized by erratic variation among litter size means with general tendency to parallelism in response to dose over all litter sizes.

An analysis of the effects of TEM dose on variances revealed interesting patterns for a number of traits. Those that confirm predictions based on earlier work (or that directly refute such predictions) are illustrated in Table 10.

One of the most interesting patterns is that illustrated by variance about the time of development of the righting response. In the F_1 generation the variance pattern for males follows that predicted on the basis of earlier work with body weight in that it shows a reduction in variance for the three experimental groups relative to that of the male control. None of these differences is significant, however. Remarkably, females show precisely the reverse pattern with all three experimental groups showing a higher variance than that of controls. Two of these

comparisons are significantly different based upon variance ratio comparisons. The pattern in the F_2 generation is precisely reversed with males, again, following the pattern predicted on the basis of body weight variation, with an increase in all experimental groups relative to the variance of controls, and one comparison producing a highly significant F value. The remarkable pattern continues in that females are precisely the reverse of males and of what they exhibited in the F_1 generation, with the control variance being greater than any experimental variance and with two of the three comparisons possible showing statistical significance. While it is possible that this peculiar pattern is a matter of chance, it is also possible that it is real, in which case the potential as a diagnostic trait is very great for righting response development.

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Table 10. Variances of (1) time of development of the righting response and (2) body weight at weaning in the F_1 generation, and of the (3) time of development of righting response and (4) incisor emergence, (5) of body weight at weaning and (6) tail length at seven weeks in F_2 generation progeny of three experimental and a single negative control group showing effects of TEM upon spermatogonia. Indications of statistical significance pertain to comparisons with appropriate controls and are based upon variance ratios.

		F_1				F_2			
Dose (mg./kg.)		0	.075	.15	.30	0	.075	.15	.30
Righting Response	♂	1.93	1.80	1.88	1.44	1.49	1.74	2.25**	1.72
	♀	1.61	2.22*	1.71	2.34*	2.25	1.88	1.42**	1.54*
Incisor Emergence	♂					1.17	.88*	1.17	.90
	♀					1.21	1.06	.86*	.96
Body Weight	♂	6.05	3.39**	4.54	4.12*	5.81	5.76	4.67	4.45
	♀	5.43	2.99**	4.88	4.45	5.38	3.53*	3.92*	3.46**
Tail Length	♂	11.22	13.47	10.05	10.11	6.45	6.20	5.34	6.00
	♀	12.39	11.22	16.73	16.16	8.53	5.06**	5.62*	7.67

* $P < .05$
** $P < .01$

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Data for the other developmental trait, time of emergence of upper incisors, appears to parallel the variance pattern of females for righting response development and other F_2 generation responses in spermatogonial treatment. While not completely consistent, in the F_2 generation all experimental variance except one are lower than controls for both males and females, and two of these differences are significant.

In the one exception, that for males in the middle dose TEM group, the variances of control and experimental are equal.

The data on variances of body weight are particularly interesting in that they offer confirmation for the prediction that mutagenic agents tend to produce a reduction in body weight variance in F_1 progeny of mutagenized males. In all comparisons between experimental groups and controls, for both males and females, body weight variances are lower than appropriate controls, and three of the differences are significant.

In the F_2 generation, the patterns predicted from work with ethyl methanesulfonate and x-ray as mutagenic agents, are precisely reversed for TEM. Experimental groups show uniformly reduced variances with respect to their controls, and all of these differences are significant in the female comparisons. The present most acceptable hypothesis to account for F_2 generation increases in variance in experimental groups following mutagen treatment of spermatozoa is that interaction between mutagenized F_2 progeny genotypes and mutagenized F_1 maternal genotypes produces an increase in variance. Reductions in variance in F_1 generation progeny of mutagenized males, under this hypothesis, would result from the buffering effect of mutagen induced polygenic heterozygosity in the F_1 young in the absence of interaction of mutagenized maternal and progeny genotypes. The simplest hypothesis to account for the results of spermatogonial treatment in our TEM study is that the mutations induced by TEM in spermatogonia may be sufficiently minor in effect so that a deleterious interaction does not occur between experimental series F_1 females and their F_2 progeny. Thus, the buffering effect of mutagen induced polygenic variation may be sufficient to reduce variance in both F_1 and F_2 generations. If this hypothesis is correct, the usefulness of this particular tool in the characterization of the effects of mutagenic agents is great.

It will be recalled that variance distribution patterns for TEM induced polygenic mutations in spermatozoa exhibited the predicted reduction in body weight variance in the F_1 generation followed by a variance increase in the F_2 progeny. This result is consistent with the present hypothesis and suggests that the mutations produced and remaining unrepaired in spermatozoa include an array of greater magnitude and of more deleterious effect than those induced in spermatogonia, the more serious of which are likely to be eliminated or repaired.

The variance patterns found for tail length in the F_1 generation, which usually appear to parallel very closely body weight patterns, do not do so here. Two of the three experimental variance values in females and one of three in males exceed those for controls. However, none of the differences was significant.

In the F_2 generation, the effects of treatment on the variance on tail length were similar to the effects observed for body weight. The variance values for all experimental groups were less than those of appropriate controls. In two of these comparisons, both in females, the differences were significant. Tail length did not appear to be as broadly useful as body weight in the overall. However, the parallelism between the pattern of change for tail length and body weight in both

means and variances offers additional evidence of the reality of the differences found in body weight mean and variance patterns, if any were needed.

Discussion. The most surprising negative result obtained in view of the results from earlier studies is the absence of any significant effects of mutagenic agent on sex ratio. In some cases the sex ratio data approached significance. However, the patterns emerging made very little sense. We will continue to study these patterns for implications that may have eluded us to the present.

Another surprising result is the lack of an effect on number carried to weaning, which has been a very good indicator in F_2 generation litters particularly in x-ray studies. We will also continue to study these data for other indications of an effect.

Of the traits studied for the first time, incisor emergence time shows the least promise, and it will be recommended below that a trait be substituted for this in the experiments of the coming year.

We will continue to carry out the spectrophotometric analysis of cholesterol levels and to analyze the resulting data so that they may be reported in the next semi-annual report.

In regard to plans for the coming year, following discussions with the project officer, it is proposed that the mutagenized spermatocyte experimental group be eliminated, and that the numbers of mice employed in this group in the past year be redistributed over other groups, particularly the treated spermatozoa group in order to increase the numbers of F_1 young, which were quite seriously depleted in the high TEM dose groups by dominant lethality. It is felt that the increase in the numbers of F_1 young available will be necessary to establish statistically some of the important results implied by our data in the past year.

It is also proposed that the antibiotic terramycin be administered uniformly to all females rearing young, control and experimental, F_1 and F_2 generation, nine days after the birth of a litter. It is our experience that the addition of antibiotic to the water of females rearing young counteracts virtually completely the effects of a disease, presumably bacterial in origin, that caused the death of significant numbers of females in our studies. While the possible mutagenic action of the terramycin itself is a concern, it would be administered well after the birth of litters and therefore could not affect the gametes from which they develop. Further, the small quantity and limited duration of administration of antibiotic could hardly lead to significant mutagenic effects. Finally, antibiotic would be administered to control and experimental groups alike, and any effects could be partitioned out as error variance in our statistical analyses.

It is suggested that the substitution of new traits for those which proved to be not too promising in studies of the past year be considered. It is recommended that brain weight after maturity be substituted for time of incisor emergence which proved to be of relatively little diagnostic value.

It is further recommended that the substitution of a behavioral trait, perhaps

some measure of physical activity, be considered in lieu of the righting response trait which showed little of interest in the study of treated spermatozoa in either generation. Inasmuch as this trait proved to be of considerable interest potentially in the mutagenized spermatogonia group, it might be well to continue to employ it for this group only. This possibility will be explored with the contract officer before decision.

Finally, the question as to the usefulness of cholesterol level as a quantitative trait in this particular kind of study should be reserved until the data are in and analyzed. It is recommended that, should cholesterol level prove to be of little value, white blood cell count be substituted as a trait in the coming year's experiment. Dr. John B. Favor, Research Associate on the project, has had prior experience with the white blood cell count as a quantitative trait and, in fact, has found differences due to the action of a mutagenic agent.

Everything considered, it is felt that the results of the first year's experiments provide strong support for the continued effort to develop a mutagenic assay based upon polygenic mutations involving the use of quantitative traits. In particular, the body weight trait has been shown to be reliable and to have considerable potential as an indication of mutagenic effect. Both tail length and hematocrit level have been found to show statistically significant variation due to mutagenic action, and both may prove to be quite useful in detecting genetic damage. Finally, the unusual patterns of sexually dimorphic variance differences in the righting response trait, in experiments involving treated spermatogonial cells, introduce a new and exciting possibility which should be explored.

Data from dominant lethal parameters have clearly indicated a consistent dose response effect as expected for spermatozoan treatment, and have failed to show an effect where one would not be expected in spermatogonia. Our results with treated spermatocytes were equivocal, but very probably reflect the mutagenic/toxic action of the mutagenic agent employed. Perhaps the most significant overall observation is that, while the dominant lethal effect of TEM at all doses on spermatogonia was undetectable, effects on quantitative trait means and variances have indicated for a number of traits, the mutagenic action, of TEM upon spermatogonia.

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Respectfully Submitted

John W. Crenshaw, Jr.
Project Director

G-32-621

REPORT OF PROGRESS FOR RESEARCH CONTRACT NIEHS NO. NO1-ES-5-2135

June 25, 1976 through December 24, 1976

The experiment proposed for the second year involved a repetition of those aspects of the experiment of the first year that appeared to require verification or substantiation. It had been proposed that traits that appeared to be without merit be omitted and that additional prospectively valuable traits be tested. It was also anticipated that sample sizes might be altered if dictated by statistical analyses of the results of the experiment of the first year, and that the mutagenic agent itself might be replaced if desirable.

Following discussions with the Project Officer, Dr. Eugene Soares, triethylene melamine (TEM) was selected as the mutagenic agent and the same agent has been employed in the experiments of the second year. Dosages selected for administration, .075, .15 and .30 milligrams per kilogram of body weight, were also employed in the experiments of the present year. As anticipated, sample sizes have been altered somewhat as dictated by the results of the first year, and there has been some substitution of traits based upon initial results. Finally, one of the germ cell stages tested (spermatocyte) in the first year has been eliminated from the present year's experiments in the interest of increasing the sample size for the other groups. It was possible to eliminate this group because there was negligible, to slight response of all traits to all treatments at this stage. Treatment of both spermatozoan and spermatogonial stages revealed promising results, and treatment of both stages is continued in the experiments of the present year.

In the experiments of the first year, the effect of TEM on F_1 and F_2 generation progeny was analyzed for eight specified traits. One of these, age of eruption of incisors, showed little promise and has been eliminated from the present experiment. Another of the original traits, serum cholesterol level at seven weeks of age, proved to be very time consuming, and a decision has not yet been made whether analyses will be continued in the present year's experiment. We are still in the process of completing the spectrophotometric analyses of samples taken last year. However, analyses run to date for F_1 generation progeny have revealed no significant differences. We are continuing to take serum samples (which are deep frozen for future analysis) should it be determined that the trait is, in fact, worth the effort. The remaining six traits of the initial experiment are being analyzed again in the present year. These include (1) young per female carried to weaning, (2) age of development of the righting response, (3) mean and variance of body weight at weaning, (4) length of tail at seven weeks of age, (5) sex ratio, and (6) hematocrit at seven weeks. In addition, we have added a seventh trait, which would be categorized as behavioral in nature, number of fecal pellets deposited in a five minute period, and (8) a morphological trait of high heritability, brain weight at 13 1/2 or more weeks of age. (For males and unproductive females, brains will be weighed at between 13 1/2 and 14 1/2 weeks of age. However, for productive females brains will be weighed after litters have been weaned at about 18 to 20 weeks of age.)

The experiments of the present year have proceeded satisfactorily with

a minor problem relating to an unknown presumably bacterial illness, in the first shipment of mice, which reduced productivity of the first replicate males in both spermatozoan and spermatogonial F_1 matings. While males were affected primarily in regard to fertility, there was some lethality among females. It is felt that the numbers of F_1 generation young available for the establishment of F_1 crosses for mutagenized germ cell stages is adequate to provide useful information. The first F_1 crosses for both replicates of the young of spermatozoan treated germ cell stages have been established. For the first replicate the matings have already been separated and for the second, mates are due to be separated prior to submission of this report.

Design. In the experiments of the present year, parental females serving as parents for determination of the effects of different germ cell stages were assigned to temporally separated replicates A and B, which were mated to males which had been exposed to mutagenic action immediately prior to mating, thus involving mutagenized spermatozoa, while groups C and D represent groups of females mated to males which had been exposed to mutagenic action over seven weeks prior to establishment of matings, thus involving germ cells mutagenized as spermatogonia. According to original plan a total of 216 male mice were treated at about 11 weeks of age and mated immediately with 648 females for a period of one week. These matings were carried out in two separate replicates, A and B, involving equal numbers of females, conducted approximately three weeks apart. Upon separation of the sexes, males were housed with females not involved in the experiment for a period of 6 1/2 weeks. Males which had been involved in the group A matings were then mated with replicate C1 females for a period of one week then with group C2 females for a second week. Similarly males which had been involved with group B matings were then mated with replicate D1 females for a period of one week followed in immediate succession by mating with group D2 females for a second week.

The spermatogonial replicates were subdivided into two mating periods due to the unequal numbers of males in the different treatment doses. The adjustment of male numbers in the different doses was designed to compensate for dominant lethal effects with increasing dose in the spermatozoan stage matings. Since there is inappreciable dominant lethal effects in the spermatogonial stages, equal numbers of females should be mated in the treatment doses. In order that all treatment groups be handled the same, the number of males in each of the dose groups was reduced to the number treated in the smallest dose group (control). To mate all females, this group of males was required to service approximately six females each. Therefore, spermatogonial groups C and D matings were separated into two one week mating periods in which each was mated to three females. When parental generation males were removed from the last matings in which they were to be involved, they were destroyed.

As in the experiments of the first year, females were isolated from males and other females eighteen days after initiation of matings to prevent post partum matings and to avoid confusion of assignment of young to a particular litter or a litter to a specific female. Young were marked for identification and the sex recorded at birth. Subsequently data on the time of development of the righting response was recorded. Approximately 22 days after males had been removed from contact with females in a given group, autopsy of unproductive females for uterine scar data was initiated.

Young were weaned at four weeks of age. At weaning the young were permanently marked by ear punching, and the sex and body weight were recorded. The fecal count portion of the open field test was conducted on mice at five weeks of age. At approximately seven weeks of age, tail length was recorded and a blood sample was obtained, hematocrit level recorded and the plasma frozen and stored for cholesterol level determination, should that be determined to be desirable, at a later date.

F_1 matings for the spermatozoan treatment groups were established with individuals between eleven and twelve weeks of age; sibling pairs were precluded. The design calls for F_1 pairs to remain together for two weeks at which time males are to be removed and their brain weights recorded prior to discarding. At the present time, males involved in the A replicate crosses have been separated from females and their brain weights recorded. Males involved in replicate B crosses are scheduled to be separated and their brain weights recorded prior to the completion of this report.

Methods. Parental males and females of replicate A were received from the Jackson Laboratory at ten weeks of age on August 4, 1976, as ordered. Males and females were housed five to a box, sexes separate until matings were established nine days later. Triethylene melamine was administered by intraperitoneal injections in Hank's Balanced Salt Solution prepared immediately prior to injection. As in the first experiment, a series of male mice were weighed to determine body weight, and all males within a dose group received the same quantity of carrier and TEM irrespective of individual variation in body weight. All mice received .25 cc of solution by injection, controls receiving the same quantity of carrier only. Injections were administered to replicate A males on August 13, 1976, and matings were established, three females to each male, immediately after injection.

Replicate B males and females were received from the Jackson Laboratory on August 13, 1976. Males were treated on September 1, 1976 and matings established immediately afterwards. This replicate was handled in precisely the same manner as the first. All males treated survived for a period of 24 hours and until the completion of the portions of the experiment involving them.

For purposes of recognition males of replicates A and B were numbered respectively 1 through 109 and 501 through 609. Females were similarly ear punched for identification and were numbered for groups A and B respectively 110 through 437 and 610 through 937.

As indicated earlier, the numbers of pairs in each of the treatment groups were adjusted to compensate for the reductions in number of young expected for the higher dose rates. Employing the results of the first year, it was determined that the total number of males assigned respectively to the control, .075, .15, and .30 mg/kg groups would be 30, 30, 50 and 106. Males to be assigned to each of these groups were selected at random, and approximately equal numbers of males were included in A and B replicates. Similarly, females were assigned randomly to the different treatment groups in appropriate numbers. Following establishment of replicate A and B crosses, cages were labeled in such a way as to preclude group identification.

Replicate A matings were established on August 13, 1976; males were removed on August 20, 1976, and placed with females not involved in the experiment in an effort to maintain a normal level of sexual behavioral activity in the males. Approximately ten days later, in expectation of production of litters, trios of mated females were separated, one to a box. Replicate B matings were established on September 1, 1976; males were removed on September 8, 1976. Approximately ten days later, trios of mated females were separated, one to a box.

Females which were to be involved in matings to test the effects of treated spermatogonia were also handled in two replicates of 324 females each. The same males that had been involved in replicate A of the spermatozoa testing matings were assigned to replicate C in the spermatogonial matings. Similarly, males which had been involved in replicate B spermatozoan matings were assigned to replicate D of the spermatogonial matings. As described above, because of the small number of males involved in the control and lowest TEM dose groups of the spermatozoa test matings, it was necessary to divide the females within replicates into two groups of equal size to be mated in sequence, three females per male.

Females of replicate C were received from the Jackson Laboratory on September 22, 1976, at 10 weeks of age and numbered 1001 to 1332 by ear punch and housed five to a box until matings were established. Half of these females were paired with males on October 4, 1976. After one week, on October 11, 1976, these males were transferred to matings involving the remainder of the group C females. One week later, these second matings were terminated on October 18, 1976. Using identical procedures, females involved in the replicate D matings were received from the Jackson Laboratory on October 6, 1976 and numbered 1401 to 1726 by ear punch. These were divided into two groups of equal size, one of which was mated with males which had been involved in the replicate B matings on October 18, 1976. These matings were terminated one week later on October 25, 1976, at which time the males were transferred to matings involving the balance of the replicate D females. These matings continued from October 25, 1976, until November 1, 1976.

Each of the four treatment groups was represented by fourteen males in each of the replicates, C and D, for a total of 112 males. The 324 females of each of the two replicates were assigned randomly to males of the different groups, three females per male. For each treatment group one male was assigned only two females to compensate for the lack of sufficient females to provide three for every male. Following termination of the final matings, males of the parental generation were destroyed. Subsequently, females were isolated, one to a box, in anticipation of production of litters.

As F_1 young were produced, they were assigned temporary numbers, within litters, and this number was coded for identification by toe clipping. Subsequently, the young were weaned at four weeks of age, a permanent number was assigned, and the animals were identified by coded ear punching. Sex was recorded at birth and again at weaning (four weeks) along with body weight, the fecal count portion of the open field test was done at five weeks, and at approximately seven weeks the tail length was recorded and the blood sample was obtained. Hematocrit level was determined and the plasma frozen and stored in the event cholesterol level determinations were desirable.

F_1 matings were established with individuals between 11 and 12 weeks of age. Males and females of a pair were randomly selected except that sibling pairs were precluded. Experimental design calls for pairs to remain together for two weeks at which time, the brain weights of the F_1 male parents will be recorded before they are discarded. A total of 108 females were mated in F_1 generation crosses of replicate A. These matings were established on November 22, 1976 and were terminated on December 6, 1976. In replicate B, 170 F_1 females were mated on December 9, 1976. These matings were terminated on December 23, 1976.

In spite of our effort to compensate for dominant lethality the number of young produced by the replicate A females mated to males which had received a .30 mg/kg TEM dose was very low. Replicate A males continued to be of rather low productivity in group C matings. Fortunately replicate B males were not similarly affected, and efforts to compensate for dominant lethality in high dose groups was effective. The high productivity of replicate B matings appears to have been maintained in replicate D matings. The simplest hypothesis to account for this low productivity of replicates A and C males is that the illness which destroyed some females in the replicate A shipment, may have interacted with the high TEM dose in males to bring about reduced fertility.

A summary of litter production of different treatment groups of replicates A and B is shown in Table 1. A total of 174 litters were produced, based upon the observation of one or more young in a cage. A total of 130 of these litters had some young surviving at weaning. Of the 44 litters lost, about half were incurred in the high dose group. Interestingly, replicate A suffered a loss of five of the total of nine litters produced, the lowered total productivity possibly a result of the sickness observed in this group and alluded to above. By contrast, the females of replicate B lost about four times as many litters, 24, but this constituted a considerably lesser portion of the 52 litters produced. In all but one of the four dose groups of replicates A and B, control and experimental, more males survived to weaning than females.

Autopsy of the females of replicates A and B have been completed and preliminary results of uterine examinations will be outlined below. For the replicate A F_1 young, data have been recorded for all with respect to sex, time of development of righting response, weight at weaning, tail length and hematocrit. Brain weights have been recorded for males only. For replicate B F_1 young the same data have been recorded except for brain weights. Cholesterol samples are available for all should analysis be indicated. Analyses of these data will be presented in the second semi-annual report.

Replicate C and D series mothers produced a total of 141 litters, based upon the observation of one or more young in a cage. This represents an impressive reduction in the total number of litters produced. However, the number of young surviving to weaning in the C and D replicates, (4.4 young per fertile female) was more than sufficient to offset the greater productivity of litters of replicates A and B in which only 3.5 young per fertile female survived to weaning. Thus, at weaning, 621 young were available for F_1 crosses in the C and D replicate test of spermatogonial effect as compared with 606 young available for crosses in the F_1 young of the replicates A and B spermatozoan effect test.

Table 1. Size distribution of litters of Replicates A and B at birth and at weaning, including three experimental and one control group. Females producing these litters were mated for a period of seven days immediately following administration of treatment to male parents, and involving germ cells treated as spermatozoa.

	Litter size	0	1	2	3	4	5	6	7	8
	number of litters:									
control	at birth	52	0	2	3	7	9	8	3	3
	at weaning	55	0	2	2	8	9	5	4	2
.075 mg/kg	at birth	54	0	4	3	13	7	3	3	1
	at weaning	57	0	2	4	11	7	4	2	1
.15 mg/kg	at birth	101	6	7	10	7	6	5	2	1
	at weaning	113	0	2	10	7	6	4	2	1
.30 mg/kg	at birth	244	5	14	8	8	7	14	5	0
	at weaning	263	0	2	5	8	10	5	5	0
Total	at birth	451	11	27	24	35	29	30	13	5
	at weaning	488	0	8	21	34	32	18	13	4

Table 2. Size distribution of litters of Replicates C and D at birth and at weaning, including three experimental and one control group. Females producing these litters were mated for a period of one week seven and one-half or eight and one-half weeks following administration of treatment to male parent, therefore involving germ cells treated predominantly as spermatogonia.

	Litter size	0	1	2	3	4	5	6	7	8	9
	number of litters:										
control	at birth	120	4	1	2	3	9	4	10	4	0
	at weaning	127	0	1	1	7	6	7	4	4	0
.075 mg/kg	at birth	117	0	3	1	4	3	7	4	4	1
	at weaning	122	0	1	0	5	4	6	3	2	0
.15 mg/kg	at birth	134	1	1	4	7	12	4	6	3	0
	at weaning	140	0	1	4	6	11	4	5	1	0
.30 mg/kg	at birth	133	0	2	4	9	6	9	3	4	2
	at weaning	140	0	0	1	10	5	7	3	4	2
Total	at birth	504	5	7	11	23	30	24	23	15	3
	at weaning	529	0	3	6	28	26	24	15	11	2

Results. Compilations of the data on dominant lethality and related losses for the A and B replicates are shown in Table 3. While these data have not yet been submitted to statistical analysis, it is evident that the trends shown parallel very closely the results of the experiments of the first year. For both replicates the mean number of live scars per female decreases with increasing dose, generally, as would be expected. This trend is the more consistent if the results of both replicates are combined. In similar manner, total number born per female bearing young and the total number weaned per female carrying young to weaning show general trends in the direction of decreasing numbers with increasing dose. Reversals in this trend for the highest dose group in replicate B are not likely significant. Again, if the replicates are combined, the trends are consistent. With respect to total scars it is generally expected that slight decreases (if any) for lower mutagen doses will be followed by more dramatic decreases at higher doses. The data from the results of replicates A and B combined are consistent with this expectation. Such reductions in scar numbers at higher mutagenic agent doses are interpreted as resulting from preimplantation losses, which would be expected to increase at high mutagenic exposures. The results of the experiments of the first year showed slight, nonsignificant, increases in total scars from the control to the lowest mutagenic dose group followed by more striking decreases in total scars successively for the two higher dose groups (Fig. 1). The results of the present year's study, however, show slight decreases in total scars from the control to the lowest dose group followed by more appreciable decreases in total scars successively for the two higher dose groups. There appears to be a general reduction in total scars for all dose groups as compared with comparable dose groups in the studies of the first year. This reduction is due partly, though not entirely, to the reduction in fertility believed to be associated with the illness discussed above. In Figure 1, our estimate of preimplantation losses is based upon the assumption that for a given dose, this may be computed as total scars minus total scars of the dose group being considered. On this assumption, it is evident that the total number of losses due to mutagenic agent (i.e., the sum of preimplantation losses and postimplantation losses represented by dead scars) increases (with a proportionate decrease in live scars per female) with increasing mutagenic agent dose.

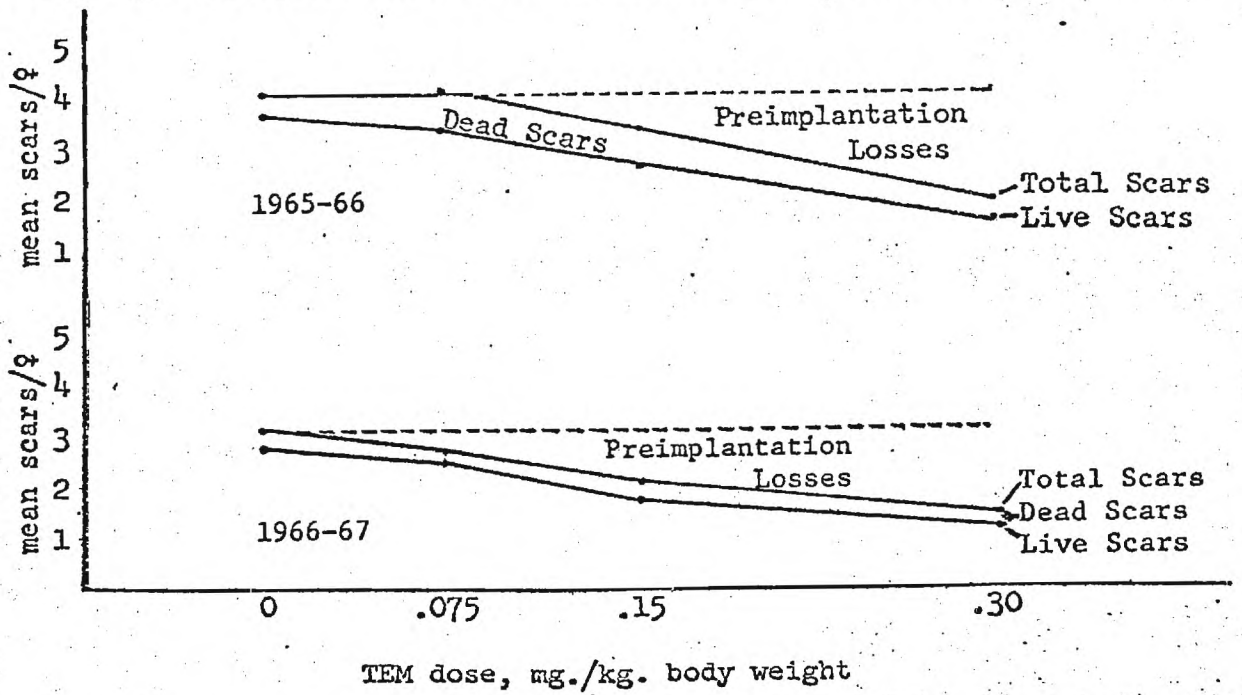
In Figure 2, the dose response curves developed by Matter and Generoso (1974) are shown with data points from our own studies superimposed for comparison. These curves are based upon living embryos in each dose group as a percent of living embryos in the control group. So that our observations are precisely comparable to theirs, we have calculated, as did Matter and Generoso, live scars per mated female for the .3 mg/kg group as a percent of controls, but for lower doses we have employed live scars among fertile females only as a percent of controls. As pointed out in the final report of the first year, given the differences that exist between our methods and theirs (i.e., our use of an inbred strain as opposed to an interstrain hybrid, and postpartum analysis of uterine scars in our study as opposed to uterine analysis at twelve to fifteen days following plugging in the Matter-Generoso study) our results are quite comparable. The slope of the dose response curve for the experiments of both the first and the second year being reported here represents a lesser rate of reduction in living embryos with increasing dose than was the case with the Matter and Generoso curves.

As was reported for the first year's experiments, we have again observed an increasing number of losses at birth with increasing dose as reflected by the differ-

Table 3. Dominant lethality parameters with standard errors and sample sizes of Replicate A and B series females and their F_1 young. Females belonging to the three experimental and a single negative control group were mated for a period of seven days immediately following treatment, thus represent effects of mutagenic agent upon spermatozoa.

	TEM dose, mg./kg. of body weight			
	0	.075	.15	.30
Replicate A				
Live Scars/ ♀	2.48 ± .43(46)	2.37 ± .42(38)	1.92 ± .26(75)	.53 ± .10(139
Dead Scars/ ♀	.30 ± .12(46)	.29 ± .09(38)	.57 ± .12(75)	.22 ± .06(139
Total Scars/ ♀	2.78 ± .46(46)	2.66 ± .46(38)	2.57 ± .31(75)	.76 ± .13(139
Total Live Births/♀	3.73 ± .54(22)	2.95 ± .47(20)	2.02 ± .36(45)	.64 ± .22 (36
Total Weaned/ ♀	4.65 ± .45(17)	3.27 ± .54(15)	3.25 ± .55(24)	1.78 ± .70(9)
Replicate B				
Live Scars/ ♀	3.35 ± .56(40)	2.83 ± .45(47)	1.44 ± .27(72)	1.81 ± .21(159
Dead Scars/ ♀	.17 ± .09(40)	.09 ± .04(47)	.25 ± .07(72)	.27 ± .04(159
Total Scars/ ♀	3.52 ± .59(40)	2.91 ± .46(47)	1.69 ± .31(72)	2.08 ± .23(159
Total Live Births/♀	4.85 ± .48(20)	3.65 ± .19(26)	2.92 ± .39(24)	3.06 ± .30(62)
Total Weaned/ ♀	4.56 ± .54(18)	4.84 ± .32(19)	2.90 ± .44(20)	3.19 ± .38(47)

Figure 1. Relationship between per female live scars, dead scars and estimated preimplantation losses for females of three experimental and a single negative control group showing the effects of mutagenic agent upon spermatozoa.



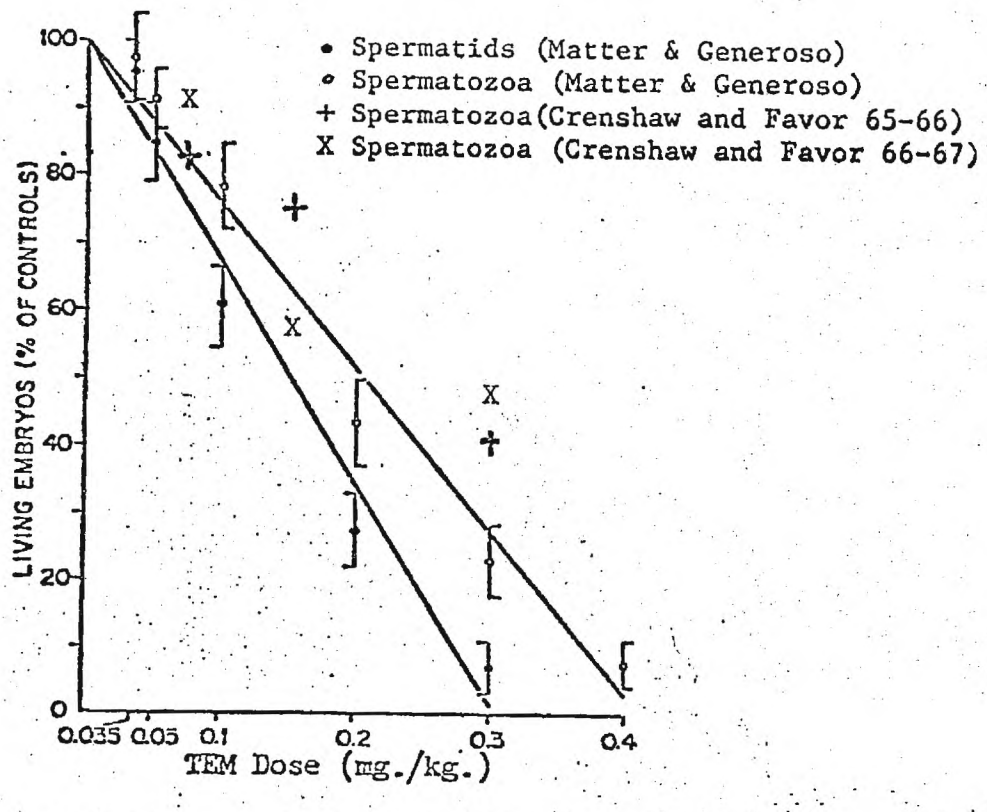


Figure 2. Effect of TEM dose on induction of dominant-lethal mutations in mouse spermatozoa and spermatids. The curves represent least-squares fits of the numbers of living embryos vs. the doses. Ninety-five percent confidence intervals are indicated by vertical bars. Taken from Matter and Generoso (1974) with results of the present study added for comparison.

ence between the number of live scars and the number of live young actually observed at birth. We have completed analyses of data for the experiments of the first year that indicate that these losses are clearly due to higher mutagen doses rather than to a litter size effect. Results of the present year will be similarly analyzed and included in a paper currently being prepared for publication reporting this interesting example of late dominant lethality.

Other analyses of data already collected and of those to be collected in the second half of the present year will be presented in the fourth semi-annual report.

Respectfully submitted:

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G-32-621

REPORT OF PROGRESS FOR RESEARCH CONTRACT NIEHS NO. NO1-ES-5-2135

December 25, 1976 through June 22, 1977

This document includes preliminary analysis of data acquired in the experiment of the second year of the referenced research research contract, and involved repetition of the first year's experiment so that observations could be confirmed and new prospectively valuable traits could substitute for traits which appeared to be poor indicators of the mutagenic effects of treatment. As in the first experiment, triethylene melamine (TEM) was the mutagenic agent employed. Dosages remained the same: 0.0, 0.075, 0.15 and 0.30 milligrams per kilogram of body weight. However, the number of matings set in each dose group was altered to compensate for the dominant lethal effects of treatment in the high dose groups. The number of matings in the higher doses was increased to produce a similar number of F_1 progeny in all dose groups. TEM treatment effects were tested on spermatozoa and spermatogonial stages of spermatogenesis.

In the first year the effects of mutagenic agent on spermatozoan, spermatocyte and spermatogonial stages were tested. Results of this experiment suggested that little of interest would be gained by repeating investigation of the effect of mutagenic agent upon the spermatocyte germ cell stage. Response of all traits to all treatments at this stage was negligible. However, treatment of both spermatozoan and spermatogonial stages revealed promising results, and treatment of both stages was continued in the experiments of the second year.

The quantitative traits studied to determine the effect of mutagenic treatment on F_1 and F_2 progeny included (1) age of development of the righting response, (2) body weight at weaning, (3) the defecation portion of the open field test, (4) tail length at seven weeks, (5) hematocrit at seven weeks, and (6) brain weight at 13 1/2 or more weeks of age. In addition, productivity traits were studied to determine if treatment was effective. These traits include (1) per cent dead implantation scars, (2) total implantation scars, (3) per cent females born, (4) total born, (5) per cent females weaned, and (6) total weaned. The productivity traits (1) and (2) determined by inspection of uterine scars were collected in the F_1 generation only (inspection of parental generation females) while the remaining productivity traits (3) through (6) were collected in the F_1 and F_2 generations (inspection of parental and F_1 generation females). Two of the traits investigated in the first year, age of eruption of incisors and serum cholesterol levels, showed little promise and were not included in the experiments of the second year.

In using a technique to estimate induced dominant lethals which allows progeny to be born and weaned, an estimate of late losses can be made (from the difference between the number of scars indicative of live embryos and the number of live born). In a number of past experiments (Crenshaw and Favor, 1976; Favor, unpublished; Soares, 1972) it has been observed that there was a treatment effect on the number of late losses. Therefore, since the data are readily available, we routinely estimate for this effect. As far as we are aware, all past dominant lethal studies have shown preimplantation and early post-implantation dominant lethal effects, but never a late post-implantation effect due to mutagenic treatment.

Finally, in response to an hypothesis suggesting that the genetic effects on quantitative traits we observe are due to induced translocations, we tested the F_2 male progeny descended from the replicate B spermatozoa treated parental males.

The experiment of the second year has been completed including all initial statistical comparisons. With the exception of an unknown health problem in the first shipment of mice, which affected male productivity in both spermatozoa and spermatogonial F_1 (replicates A and C matings), no problems arose.

Design. The experiment of the second year was conducted to determine the effects of mutagenic treatment in the spermatozoan and spermatogonial stages of spermatogenesis on quantitative traits. Each spermatogenic stage was represented by two temporally separated replicates of equal size. These replicates were designated A and B for the spermatozoan stage and C and D for the spermatogonial stage. Within each replicate, four dose groups were included 0.0, 0.075, 0.15, 0.30 milligrams per kilogram of body weight of TEM. Parental males were treated and immediately paired for one week to three females to produce the spermatozoan stage treated groups. Males were subsequently housed with females not involved in the experiment for 6 1/2 weeks at which time the males were mated to females to produce spermatogonial stage treated groups. Thus, the spermatozoan stage group A and the spermatogonial stage group C were produced by the same parental males. Groups B and D are similarly related. Precise timing of replicate matings is shown in Table 1.

Females were isolated from each other eighteen days after initiation of matings and checked daily for the presence of a litter through day twenty-one after the termination of matings. Young were sexed and marked by toe clipping for identification at birth. Starting on day five, progeny were checked for the ability to right (righting response) and were checked daily until the trait appeared. All animals were weaned at four weeks of age, at which time body weight and sex were recorded and individuals were permanently marked by ear punching. The defecation portion of the open field test was conducted on mice at five weeks of age while tail length and hematocrit measures were taken on mice at approximately seven weeks of age.

Matings of F_1 individuals to produce F_2 progeny were established when F_1 individuals were between eleven and twelve weeks of age. Crosses were set by randomly mating individuals within a dose and a replicate except that sibling crosses were precluded. Thus, four replicates were produced in the F_2 generation each represented by four dose groups. The four replicates were designated A, B, C, and D, and correspond to the F_1 replicates. (That is, F_2A includes the F_2 progeny of replicate 1 spermatozoa treated males. F_2B is the F_2 progeny of replicate 2 spermatozoa treated males. F_2C and F_2D are the F_2 progeny respectively of replicates 1 and 2 spermatogonia treated males). F_1 matings were set for two weeks. Males were separated from females of the F_1 matings, and brain weights recorded. Brain weights of females were recorded when litters of the group had been weaned.

Methods. A time course of the second year experiment is given in Tables 1 and 2.

Table 1. Time course of parental generation matings.

	Replicate			
	A	B	C	D
Mice received	8/4	8/13	9/22	10/6
Matings set	8/13	9/1	10/4;10/11	10/18;10/25
Matings separated	8/20	9/8	10/24;10/30	11/7;11/14
Mice weaned	9/30-10/6	10/19-10/26	11/22-11/26; 11/29-12/6	12/6-12/13; 12/13-12/20

Table 2. Time course of F_1 generation matings.

	Replicate			
	A	B	C	D
Matings set	11/22	12/9	1/12	2/3
Matings separated	12/6	12/23	1/26	2/17
Mice weaned	1/10-1/17	1/27-2/8	3/1-3/11	3/24-4/5

Treatment consisted of intraperitoneal injection of triethylene melamine carried in .25 cc Hank's balanced salt solution. Doses were based upon the mean body weight of a random sample of the mice to be treated. The numbers of matings set in each dose were adjusted to compensate for dominant lethal effects in the higher doses so as to produce similar numbers of F_1 progeny. The total number of males treated in replicates A and B combined in the dose groups 0.0, 0.075, 0.15, and 0.30 mg/kg of body weight was respectively 30, 30, 50, and 106. All animals were identified by a coded number so that all observations were blind with respect to dose.

Since there are no substantial dominant lethal effects in spermatogonial treatment, replicate C and D dose groups were established with equal numbers of matings. With unequal numbers of males in the dose groups established for replicates A and B, the number of males mated in each dose group of replicates C and D was equal to the number of males in the smallest dose group (0.0 mg/kg with 30 males). To mate all females, each male had to service 6 females. Thus, replicates C and D matings were subdivided into two one week mating periods.

A summary of productivity data for each replicate and dose in the F_1 and F_2 generations is given in Tables 3 and 4. In replicate A parental matings, treatment produced the expected effects on productivity data. Per cent fertile matings was reduced at the highest dose, litters born as a per cent of fertile matings was reduced at the two highest doses, and litter size at birth, litters weaned as a per cent of litters born, and litter size at weaning all decreased as dose increased.

For replicate B parental matings, treatment appeared to have reduced the per cent fertile matings at the 0.15 as well as the 0.30 doses. The number of litters born as a per cent of fertile females appears to have been affected only slightly by dose. Both litter size at birth and at weaning decreased as dose increased up to a dose of 0.15 but then increased slightly at the highest dose. Litters weaned as a per cent of litters born appeared to be reduced at the highest dose.

As expected, no differences in productivity were found among spermatogonial treated parental matings (replicates C and D). In general, per cent fertile matings was low and was probably due to the large number of females each male was required to service.

In the F_1 matings, per cent fertile matings, the per cent of litters weaned that were born and litter size at birth is reduced as dose increased in the spermatozoa treated groups (replicates A and B) but not in the spermatogonial treated groups. Finally, with the exception of replicate A in which there was the possibility of an illness affecting productivity (see semi-annual Report of Progress June 76-Dec. 76), we were successful in producing similar numbers of animals in all dose groups.

Table 3. Productivity data of Parental matings.

Rep.		Dose			
		0.0	0.075	0.15	0.30
A	Females mated	46	38	75	139
	Females fertile	22(.48)	20(.53)	45(.60)	36(.26)
	Litters born	17(.77)	15(.75)	24(.53)	9(.25)
	Litter size birth	3.73	2.95	2.02	.64
	Litters weaned	16	12(.80)	16(.66)	4(.44)
	Litter size weaned	4.65	3.27	3.25	1.78
B	Females mated	40	47	72	159
	Females fertile	20(.50)	26(.55)	24(.33)	62(.39)
	Litters born	18(.90)	19(.73)	20(.83)	47(.75)
	Litter size birth	4.85	3.65	2.92	3.06
	Litters weaned	16(.89)	19(1.00)	15(.75)	31(.66)
	Litter size weaned	4.56	4.84	2.90	3.19
C	Females mated	80	66	89	88
	Females fertile	15(.19)	16(.24)	32(.36)	19(.22)
	Litters born	12(.80)	9(.56)	27(.84)	17(.89)
	Litter size birth	4.33	3.50	4.06	4.58
	Litters weaned	10(.83)	8(.88)	22(.81)	12(.70)
	Litter size weaned	4.92	5.22	4.15	4.18
D	Females mated	78	77	83	83
	Females fertile	29(.37)	21(.27)	15(.18)	26(.31)
	Litters born	25(.86)	18(.85)	12(.80)	22(.85)
	Litter size birth	4.52	4.52	4.27	4.62
	Litters weaned	20(.80)	12(.66)	10(.83)	20(.91)
	Litter size weaned	4.24	4.28	4.00	5.00

Table 4. Productivity data of F_1 matings.

Rep.		Dose 0.0	0.075	0.15	0.30
	Females mated	35	26	38	5
	Females fertile	30(.85)	18(.69)	28(.74)	3(.60)
A	Litter size birth/mated	4.27	3.88	3.55	1.20
	Litters weaned	14(.46)	6(.33)	17(.61)	0(0)
	Litter size weaned/ fertile	2.10	1.77	2.82	0
	Females mated	34	39	24	70
	Females fertile	31(.91)	31(.79)	19(.79)	51(.73)
B	Litter size birth	4.21	3.41	3.46	2.97
	Litters weaned	16(.52)	16(.52)	8(.42)	22(.43)
	Litter size weaned	2.45	2.16	2.26	1.94
	Females mated	31	17	54	32
	Females fertile	26(.84)	13(.76)	42(.77)	25(.78)
C	Litter size birth	3.68	2.47	3.48	3.69
	Litters weaned	12(.46)	4(.31)	24(.57)	17(.68)
	Litter size weaned	2.36	1.23	2.56	3.60
	Females mated	56	34	23	46
	Females fertile	45(.80)	27(.79)	17(.74)	39(.85)
D	Litter size birth	3.64	3.44	3.35	3.76
	Litters weaned	24(.53)	17(.63)	12(.70)	25(.64)
	Litter size weaned	2.58	2.85	3.47	3.31

Results. Data on parameters associated with dominant lethality in females mated to males receiving spermatozoan treatment were provided in the Report of Progress submitted for the period ending December 24, 1976, as Table 3. Means, standard errors and sample sizes were shown separately for replicates A and B for the following measures: live scars per female, dead scars per female, total scars per female, total live births per female and total weaned per female. Data had not been submitted to statistical analysis at that time, but it was pointed out that they paralleled very closely the results of experiments of the first year. From that report, "for both replicates the mean number of live scars per female decreases with increasing dose, generally, as would be expected." Replicates were combined in Figure 1 of the previous Report of Progress to show the similarity and trend of data from the first year's experiment. As has been reported total live births for replicates A and B showed parallel results, with numbers of live births per pregnant female being inversely related to dose. These same data, in modified form, are included in this report as Table 3. Subjected to statistical analysis, it was found that there were highly significant differences ($P < .001$) in live births per fertile female between dose groups and between replicates. Variance due to interaction proved to be not significant. The numbers of live births found for each dose group in replicate B was higher than in the comparable group of replicate A. Replicate and dose mean litter size at birth is shown in Table 3.

Young weaned per fertile female showed highly significant differences due to dose ($P < .001$), but the difference between replicates was not significant nor was interaction between replicate and dose. Data for replicates A and B have been combined in Table 5. Comparable data from the experiment of the first year are included for comparison. The close similarities are evident.

Analysis of the results of variation in total scars must take into consideration the likelihood that reductions in total scar numbers may be very slight, if any, for lower mutagen doses but will be followed by more dramatic increases at higher doses, presumably due to preimplantation losses, which would be expected to increase at very high mutagenic doses. Thus when we analyze our results based upon pregnant females only, those females that suffered complete loss of litter due to preimplantation loss are excluded. These expectations are consistent with our findings. In Table 3 of the last Report of Progress, total scars per mated female were shown to decrease rather regularly with increasing dose for both replicates A and B, except that the highest dose group of replicate B showed a reversal of the trend, i.e., total scars were slightly greater than for the next highest dose. Statistical analysis of these results based upon square root transformation of total scars per mated female revealed that differences due to dose are significant ($P < .05$) while differences between replicates are not so. Interactions between group and dose were highly significant ($P < .01$), presumably due to the increase in replicate B of scars per female for the highest dose group over the next highest. An analysis of square root transformation of total scars per fertile female eliminates the significant interaction variance as well as differences due to dose, however differences between replicates remain significant. This suggests that the interaction observed was likely due to the inclusion of large numbers of females with zero scars in the "per mated female" analysis (perhaps combined with the effect of an illness in replicate A which may have affected these mean values). While total scars per mated female for replicates A and B may not be combined for statistical purposes, we have included combined weighted means for descriptive purposes in Table 5. Comparable values for the experiments of the first year are included to show the similarity. The decreases in total scars with increasing mutagen dose in the experiments of both first and second years were significant, and it is clear that the dose response curves are very similar, involving inappreciable differences between the control and lowest mutagen dose, modest reductions in total scars at the next highest dose and striking reductions at the highest mutagen dose.

In Table 3 of the last Report of Progress, dead scars per mated female revealed a pattern involving little difference between the control and lowest mutagen dose groups followed by an increase in the next highest dose group and contrasting responses in replicates A and B with respect to the highest dose group, with a remarkable drop in numbers in replicate A and a negligible increase in numbers in replicate B. However, these figures, based upon dead scars per mated female may have been affected inordinately, as were analyses of total scar variation, by the very low fertility of females in the highest dose group of replicate A.

A more useful measurement of variation in dead scars is percent dead scars of total scars, which may be analyzed either on a "per fertile female"

or a "per mated female" basis. We have carried out statistical analyses on both bases. The per mated female analysis has the disadvantage that a decision has to be made as to whether infertile females should be treated as having lost all their young prior to implantation. Either approach is patently in error, but the evidence indicates that preimplantation loss may be very high for high mutagen dose groups and not very important for low dose and control groups.

Table 5. Dose responses of selected indications of dominant lethality with standard errors and sample sizes of parental series females and their F_1 young comparing results of experiments conducted in 1975-76 (I) with those carried out in 1976-77 (II).

Trait	Year	TEM dose in mg./kg. of body weight			
		0	.075	.15	.30
Total weaned/fertile	I	7.97 ± .27	4.20 ± .25	3.86 ± .32	3.74 ± .36
	**II	4.60 ± .35	4.51 ± .32	3.09 ± .36	2.96 ± .34
Total scars/mated	*I	4.01 ± .36	4.10 ± .32	3.29 ± .30	1.86 ± .25
	*II	3.13 ± .45	2.80 ± .33	2.14 ± .22	1.46 ± .14
Dead scars per cent of total scars	**I	10.4	19.0	21.3	20.9
	**II	7.8	6.3	19.4	17.0
Late losses per cent of total scars	I	21.86 ± 4.59	26.27 ± 5.02	44.29 ± 5.52	54.50 ± 7.10
	II	25.44 ± 5.27	36.33 ± 6.02	39.45 ± 5.36	41.90 ± 4.42

* $P < .05$
** $P < .01$

In any event, the statistical analyses, carried out after arc sine transformation of per cent dead scars of total, per mated female, were not very satisfactory even though effects due to variation in mutagenic dose are highly signigicant. However, variance due to interaction between replicate and dose, probably due to the same factors discussed above, is also highly significant.

Analysis on a per fertile female basis is more satisfactory, with differences due to dose and replicate each being highly significant, but differences due to group X dose interactions negligible.

Data on per cent dead scars of total are the most meaningful and therefore persuasive of the data in the study that the mutagenic agent administered has been effective. While, again, we have submitted our data to factorial ANOVA after arc sine transformation by replicate and dosage froups, for descriptive purposes it is useful to combine the two replicates to produce weighted group mean values for the separate dose responses. These are shown in Table 5 with the results of the study of the first year also shown for comparison. Indications of statistical significance are based upon ANOVA of arc sine transformed percentage values.

As noted in the last semi-annual report, in the results of both the first and the second year's experiments, we observed an increasing number of losses at birth with increasing mutagen dose. This loss was computed as the difference between the number of uterine of live scars and the number of live young actually

observed at birth. We have now completed analyses of the data for the experiments for both years. The results of the first year's experiment, as reported, revealed significant differences between dose groups, and are supported by the mean distribution of the experiments of the second year. A factorial analysis of variance of the differences between dose groups with respect to percentage late dominant lethal losses of total scars for both years reveal that the differences were highly significant ($P < .001$). Similarly the differences between the experiments of the first and second year were highly significant ($P < .001$); interaction between experiment and dose was not significant. Results of an ANOVA after arc sine transformation of data resulted in similar conclusions except that the probability of chance error was increased ($P \sim .03$). In order to reduce the chances of committing a Type I Error, our data were modified by a correction factor to determine if misclassifications of dead scars as live scars could account for the differences observed. Although, differences between dose groups were no longer significant, it was found that the correction factor could not account for the variation observed, and that the basic pattern of increase in late dominant lethal losses as a function of increasing mutagen dose was still evident. Late dominant lethal loss means are shown (Table 5) as a percent of total scars for the separate dose groups for the experiments of the first and second year.

All group B F_2 males (154) were tested for semi-sterility to determine if they included translocation heterozygotes. A stock of randomly mated females with high productivity (CD-1, Charles River Breeding Laboratories) was used to test males. Each male was paired to two females. Eighteen days after matings were set, females were sacrificed and their uterine horns examined for live and dead embryos. Matings were set on 4/15/77 and 4/18/77 and females dissected on 5/2/77, 5/3/77, and 5/4/77. In those cases where the results indicated the possibility that the male (12) might be a translocation heterozygote, additional matings (two females) were set on 5/6/77 and females dissected on 5/23/77. No male had fertility data which would classify it as a translocation heterozygote. One male proved to be sterile in all matings and is being retained for chromosomal examination. However, it is unlikely that a translocation causing such infertility could have survived to the F_2 generation.

With respect to dominant lethal parameters for females producing young which were the products of fertilization by spermatozoa exposed to mutagenic treatment in the spermatogonial state, there were significant differences between dose groups in replicate C ($P < .05$) only with respect to percent dead scars of total. The high variance between groups was due largely to relatively high dead scar percent (14.3%) in the lowest mutagen dose and a relatively low percent dead scars (4.6%) in the highest mutagen dose. Replicate D exhibited no significant differences with respect to this trait, nor was there any similarity in general to the pattern exhibited by replicate C. Since there is no simple biological explanation to account for the pattern in replicate C it is felt that unusually great chance variation may be involved. In the experiments of the first year, there were no significant differences observed among comparable groups for any dominant lethal measure.

Quantitative traits. The primary purpose of the experiments conducted over the past two years has been to determine the usefulness of quantitative traits as indicators of mutagenic activity. In studies of the first year and in earlier experiments our results have indicated that mutagen induced polygenic mutations, that is mutations of minor effect, may have effects both upon the means and the variances of some quantitative traits. Our results of the first year indicated that some traits were particularly useful while others were not, and that differences due to mutagenic treatment might be detected in either the F_1 or F_2 generation progeny of mutagenized males. While information from either generation may be of interest as an indicator of genetic damage following mutagenic treatment, in general effects observed in the F_2 generation are more certainly attributable to a genetic basis than those of the F_1 generation. Nevertheless, genetic causation is likely even if it cannot be conclusively demonstrated to be a cause of observed F_1 generation effects.

Our results of the second year have, in some cases, supported conclusions arrived at in the first year. In other cases, traits were not found to be so broadly useful as might have been hoped. In still others, traits which did not appear to be particularly useful in the first year's experiment proved to be of potentially greater usefulness than had been anticipated. In general, our decision to divide the total experiment into two successive replicates reduced the potential to demonstrate significant differences within each replicate. Unfortunately, this potential was even more adversely affected by an illness which affected our replicate A. Even so, this experience can be useful in providing some idea as to the extent of environmental variation acting upon the traits investigated. Finally, sexual dimorphism was found with respect to every trait investigated thus necessitating factorial analyses or comparisons within a given sex.

In the analyses to follow, we will first deal with the effects of mutagenic treatment upon the spermatozoan stage of spermatogenesis in the F_1 and F_2 generations. Subsequently we will consider the effects of mutagen treatment upon the spermatogonial stage of spermatogenesis in the F_1 and F_2 progeny.

Effects of Mutagen on Spermatozoa: F_1 Generation

In broad overview, results of the second year's experiments (Experiment II) were similar to and support those of the first year (Experiment I) with respect to hematocrit where it was evident that increases with increasing dose occur in a roughly linear manner. Differences between the two replicates of Experiment II are not significant so that they may be combined.

With respect to body weight and tail length, results tended to be in the same direction in Experiment II as in Experiment I. However, differences due to dose were not significant, and individual experimental face value means were not always greater than those of their controls as predicted.

Data were obtained for the time of development of the righting response

in Experiment II primarily because of data suggesting an interesting effect in the F_2 generation of Experiment I. However, data obtained for F_1 generation young in Experiment II proved to be quite interesting in their own right. When the effects of litter size were partitioned out, there remained highly significant differences due to both dose and sex. Since there were no significant interactions between group and dose effects, the two replicates could be combined for analysis. In general, there appeared to be a decrease in rate of development with increasing mutagen dose.

With respect to two new traits analyzed, the defecation portion of the open field test and brain weight at 13 1/2 or more weeks of age, no significant differences were found due to mutagen dose. With respect to the defecation portion of the open field test it was found that there was significant variation in this trait due to litter size ($p < .05$) and there was a highly significant difference between sexes ($p < .01$). However mean differences between dose groups were very slight and showed no suggestion of any of the several types of patterns that have been evident in data with respect to other traits. Accordingly, we will eliminate the defecation portion of the open field test from future experiments.

The situation with respect to brain weight is more interesting. While differences due to dose were not significant ($P \sim .21$), a modest increase in the sample size with similar variances and distribution of means might well lead to a demonstration of significant differences. An interesting pattern of mean distribution also emerged in that, without exception, the brain weights of all progeny of treated males was greater than the appropriate control. These results will be treated in greater detail below, and it is planned that future experiments will continue to explore the usefulness of mutagen effects upon brain weight.

In final analysis, hematocrit appears to be a most useful trait for detection of mutagenic effect in F_1 generation progeny involving mutagenized spermatozoa. Although the magnitude of the differences is not as great as has been found for some traits, relatively low variation within groups would seem to make mean differences very useful as indicators of genetic effect. Whether hematocrit variance will prove to be of usefulness or not will depend upon additional evidence. Very likely it will be necessary to employ much larger sample sizes than have been employed to date to detect effect upon variance.

Body weight. The exciting results of Experiment I were not uniformly confirmed in Experiment II. As expected, litter size was shown to have a highly significant effect upon body weight. After partitioning the effect of litter size, highly significant differences were found to exist between replicates and between sexes. While differences between dose groups were not significant ($p \sim .16$), in general the mean body weight of progeny of mutagenized males were greater than those of controls as in Experiment I. The only exception to this was the high mutagen dose group of replicate B. Mean body weights for Experiments I and II sexes (and replicates of Experiment II) combined, are shown in Table 6.

The distribution of variances in Experiment II is in remarkable contrast with those of Experiment I. In the latter experiment variances of the progeny of mutagenized males were uniformly lower than those of controls, more often than not significantly so. By contrast, in the experiments of the present year

Table 6. Means of hematocrit at seven weeks, body weight at weaning, tail length at seven weeks, time of development of righting response, and brain weight at 13 1/2 or more weeks of age in F_1 generation progeny of three experimental groups and a single negative control for two experiments showing effects of TEM upon spermatozoa. Replicates for Experiment II are combined. Means over solid lines at the same level, whether continuous or connected by dashed line, are not significantly different by multiple range comparison.

Experiment	Dose (mg./kg.)	0	.075	.15	.30
I	Hematocrit ♂ & ♀	48.24	48.42	48.67	48.79
II	♀	46.05	46.27	46.28	46.53
	♂	46.79	46.97	47.26	47.16
I	Body weight ♂ & ♀	13.06	13.54	13.94	14.05
II	♂ & ♀	14.46	14.66	14.84	14.44
I	Tail Length ♂ & ♀	69.75	70.82	70.80	70.58
II	♂ & ♀	73.99	74.26	74.30	74.42
I	Righting Response				
	♂	8.10	8.33	8.20	8.30
	♀	8.03	7.89	7.86	8.74
II	♂	7.52	7.20	7.81	7.96
	♀	7.28	7.23	7.44	7.31
II	Brain size ♂ & ♀	419.86	422.59	422.03	422.16

Table 7. Variances of hematocrit at seven weeks, body weight at weaning, tail length at seven weeks and time of development of righting response in F_1 generation progeny of three experimental and a single negative control group showing effects of TEM upon spermatozoa. Indications of statistical significance pertain to variance ratio comparisons with appropriate controls.

Experiment	Dose (mg./kg.)		0	.075	.15	.30
I	Hematocrit	♀	1.74	1.64	3.20**	1.56
		♂	2.10	2.37	3.80**	2.16
II		♀	2.65	2.90	2.67	3.09
		♂	2.34	2.32	1.90	1.49*
I	Body weight	♀	5.34	3.50*	4.28	2.02**
		♂	7.62	4.45*	6.81	2.46**
IIA		♀	1.77	2.43	3.06	1.56
		♂	2.34	3.92	2.43	1.30
IIB		♀	2.01	2.25	3.50	3.42
		♂	2.82	2.82	3.06	3.92
I	Tail length	♀	13.03	9.80	9.24	6.71
		♂	9.67	9.99	12.18	4.04**
IIA		♀	4.37	3.88	5.52	2.56
		♂	5.34	7.24	7.40	2.50*
IIB		♀	9.00	4.38*	4.20*	4.28*
		♂	4.45	4.54	4.37	4.16
I	Righting response					
		♀	1.66	2.28	2.82*	2.10
		♂	2.46	2.59	2.72	1.74
II		♀	.95	1.47*	1.09	1.04
		♂	.82	1.05	1.11	1.01

* $p < .05$
** $p < .01$

the reverse is generally true, the only exception being the high dose group of replicate A in which variances for both males and females were below those of controls. However, no significant differences were observed between any experimental group and its appropriate control. Perhaps the reduction in numbers associated with splitting the total sample into two replicates has made it difficult to reveal meaningful variation in body weight variances.

Hematocrit. Variation in hematocrit was analyzed by factorial ANCOVA by mutagen dose, replicate, and sex with litter size. Effects due to litter size and replicate were not significant. Effects of mutagen dose were significant ($P < .05$) and highly significant sexual dimorphism was found ($P < .001$). This latter result is in contrast with those of the results of the first year where differences between sexes (in the same direction as in Experiment II) were not significant. Mean values for males and females combined in Experiment I and tabulated separately for Experiment II are shown in Table 6. It is evident that F_1 progeny of mutagenized males invariably show higher hematocrit values than their appropriate controls. In the experiment of the first year it was found that the middle mutagen dose only was significantly higher than control, but the small sample size of the highest mutagen dose may have prevented demonstration of significance which for the highest mean value observed in the comparison. In the experiments just completed, while our analysis of variance indicates that there are significant differences between dose groups, a multiple range test, which considers the number of comparisons being made, does not lead to conclusions of significance for any comparison. However, the values that most closely approach significance are those for the highest mutagen dose in comparison with control for females, and the two highest mutagen doses as compared with control for males.

With respect to variance comparisons of hematocrit, patterns that emerge are not really convincing although certain points of interest remain. In Experiment I the two significant differences that occurred were between the (high) variances of males and females of the middle mutagen dose and the (low) controls. In other comparisons with controls male progeny of mutagenized males exhibited higher variances, females lower. In Experiment II precisely the reverse trend is evident. Experimental variances for female F_1 progeny are uniformly higher than those of controls, and experimental variances for F_1 male progeny are without exception lower. Only one of these comparisons is significant, that for the high dose male group in comparison with the male control.

Tail length. Analysis of variation in tail length by factorial ANCOVA indicates that there is highly significant variation associated with litter size, between replicates and between sexes, but differences among dose groups are not significant. After correcting for litter size, there is a roughly linear increase in tail length with increasing mutagen dose as in Experiment I. However these differences are not significant as they were in Experiment I. Means corrected for litter size for Experiment I and Experiment II, sexes and replicates combined, are shown in Table 6.

Variances with respect to tail length in Experiment I showed, in general, reductions among female progeny of mutagenized males relative to controls (Table 7), but increases among male progeny of mutagenized males relative to controls (except for the high dose group where small sample size was a problem). In Experiment II, considering replicates and sexes separately, all female progeny of mutagenized males except one (of six groups) exhibited tail length variances of lesser magnitude than controls, and three of these were significant. Tail

length variances for male progeny exhibited no pattern in comparisons of control and experimentals, although the replicate A, high mutagen dose group was significantly lower than its control. Small sample size (N = 11) could be the responsible factor.

Righting response. As indicated above, in Experiment II, data obtained with respect to the effect of mutagenic agent on the time of development of the righting response proved to be more interesting than anticipated. It was found that there was highly significant variation associated with litter size and with sex. Differences between dose groups were also highly significant ($P < .001$). A significant dose x sex interaction was also found. Since there was no suggestion of a difference between replicates, of significant group x dose or group x sex interactions, replicates were combined for purposes of this analysis.

In the experiments of the present year, there is a general trend in both replicates for an increase in the time of development of righting response with increasing mutagen dose. Interestingly, the lowest mutagen dose appears to have no effect on the effect of reducing developmental time, but in both sexes of both replicates the high mutagen dose is associated with a longer developmental time than that of the control (and the low mutagen dose groups). The phenomenon is especially evident in females where a multiple range test indicates that the high mutagen dose is associated with a significantly longer time to development of righting response than the control group, and both high and middle mutagen dose groups have significantly longer times to development of the righting response than the low mutagen dose. Interestingly the increase in rate of development, i.e. reduction in time of development to the righting response, associated with the low mutagen dose group approaches significance in comparisons with the control. We have observed this pattern before in respect to other traits, and it may well be real.

A reexamination of the results of the first year's experiment reveals certain similarities of these patterns except that the males showed a more pronounced effect in that experiment than the females, in contrast with those of the present year. However, face value means for both males and females for the high mutagen dose groups were greater than for their respective controls. For the male group there was also observed a reduction in time of development to righting response for the low and middle mutagen dose groups below that of control. The probability that the differences between dose groups were real was sufficiently high ($p \sim .25$) so that these results were not singled out for critical discussion. The means for Experiment I and II are shown in Table 6 with replicates combined for Experiment II.

With respect to variation in variance of the righting response, there is limited variation between doses, and male variances are generally lower than those of females. In a comparison of variances with sex, replicates combined, those of progeny of mutagenized males are uniformly higher than those of controls and one of these differences is significant (Table 7). A similar trend was seen in the first year's experiment, the only exception being the variance for the high mutagen dose male group. One of the increases in variance in an experimental group over that of control is significant.

Brain weight. Brain weight was one of the traits introduced for the first time in the present year's experiment and would seem to have some promise in spite of the fact that the difference between dose groups was not significant ($P \sim .21$). It will be evident that similar differences and variance with twice the number of experimental subjects employed would produce an impressive level of significance. Of greater importance is the fact that a pattern emerges from these results that would be of interest as an indicator of mutagenic activity, if the differences observed approximate reality. Our analysis of variance indicates that brain size is not significantly associated with litter size. However, there are highly significant differences between replicates and between sexes. Interactions between group, dose and sex were not significant.

Examining the distribution of means for both replicates, sexes considered separately, the generalization that emerges is that with two exceptions (out of 12 comparisons possible) progeny of mutagenized males exhibited larger brain size than did their appropriate controls. The difference between brain size of sexes is likely due in large part to the fact that females were approximately one month older than males when their brains were weighed, in order to permit rearing of litters. Since there were no significant interactions between dose and group or dose and sex , for simplification, we have shown dose means adjusted for litter size with sexes and replicates combined, in Table 6. Variance with respect to brain weight showed no patterns of interest and rather limited variation.

Effects of Mutagen on Spermatozoa: F_2 Generation

The traits that stand out as being of interest in effects in the F_2 generation following treatment of spermatozoa include the righting response in which the pattern of distribution of means was strikingly similar to differences observed in the F_1 generation with reduction in developmental time at low doses but increase at higher doses. Again, following the partitioning of litter size effects, there remain highly significant differences due to dose. Reexamination of the results of the first year's experiments fail to reveal any similarity with the results obtained in the present year's study.

Brain weight proved to be a particularly exciting possibility for F_2 generation analysis. It was found that effects due to litter size and replicates were highly significant ($P < .001$) as were those due to dose ($P < .003$). The most evident difference was the increase in brain size associated with the lowest mutagen dose over control brain size, followed by a more or less linear reduction in size with increasing dose. Means for all of the experimental series groups remained above the control means.

While differences associated with dose in regard to tail length were not significant, they were nearly so, and patterns that emerged resembled rather closely patterns of the first year's experiments. These will not be reported upon in further detail here but it is planned that more detailed analyses will be carried out in the future to investigate the possibility of repeatable patterns that could be of usefulness.

With respect to the other traits examined, there was little of interest. The defecation portion of the open field test showed no significant differences due to dose, nor was the pattern of mean distribution particularly interesting. Similarly, differences due to dose in regard to body weight were not significant. Although, as would have been predicted from the first year's experiments, body weights for all three experimental series groups when adjusted for litter size were greater than the mean body weight of the control group. The prediction of an increase in body weight variance for experimental groups over those of controls was not confirmed by the present year's experiments. Of the ten comparisons possible, only six were in the predicted direction. Differences among dose groups with respect to hematocrit variation also proved not to be significant, nor was there any pattern of interest in the distribution of means.

In summary the time of development of righting response and brain weight would appear to be the most useful characters for F_2 generation analysis of mutagen effect upon spermatozoa. The consistency of increase in body weight in the F_2 progeny of mutagenized males over that of controls also suggests that this trait would be a useful indicator, particularly in dose response analyses.

Table 8. Means of time of development of righting response, brain weight at about 13 1/2 weeks and body weight at weaning in F_2 generation progeny of three experimental groups and a single negative control for two experiments showing effects of TEM upon spermatozoa. Replicates of Experiment II are combined. Means over solid lines at the same level, whether continuous or connected by dotted line, are not significantly different by multiple range comparison.

Experiment	Dose (mg./kg.)	0	.075	.15	.30
II	Righting response	8.53	7.88	9.05	8.24
II	Brain weight	411.97	419.32	413.95	414.45
		408.99	417.10	412.50	416.36
I	Body weight	12.79	13.19	10.87	13.00
II		12.91	13.31	13.22	13.06

Righting response. An analysis of variance of variation in time of development of the righting response revealed that there were highly significant

effects associated with litter size ($P < .001$), but differences between replicates and due to sex only approached significance ($P < .10$). Differences among dose groups were highly significant ($P < .01$). For males and females and replicates A and B there was a consistent reduction in time of development of the righting response in the lowest mutagen dose group below that of controls followed generally by a slowly increasing time of development with increasing mutagen dose. Replicates and sexes have been combined in Table 8. Had the means been adjusted for variation in litter size, linearity of the response would be more evident. The simplest interpretation of these results is that the slightest amount of mutagen induced variation has the effect of shortening developmental time appreciably. With increasing mutagen effect, i.e., genetic damage, developmental times are not so strikingly reduced. Comparing these results with those of the F_1 generation (Table 6) it may be seen that the same pattern was present except that developmental time was not shortened so noticeably at comparable doses and, at the highest mutagen doses, experimental means exceeded control group means. This interpretation is based upon the assumption that a very limited amount of mutagen induced polygenic variance may provide a buffering effect that permits more rapid development. With increasing mutagenic damage, however, the cumulative effects of deleterious mutations will gradually mask the buffering effects of those polygenic mutations with effects in the normal range of variation. Rate of development is a phenomenon closely related to fitness, and it might be predicted that other fitness traits would show a similar pattern where the direction of "bad" or "good" effect can be determined in a relatively straightforward manner.

Variance in regard to righting response showed no particular pattern. Of ten comparisons possible, exactly five experimental variances proved to be greater than their controls, but only one of these was significantly so.

Brain weight. An analysis of variance of the variation in regard to brain weight showed that there was highly significant variation ($P < .001$) associated with litter size and between replicates. Differences between the sexes were not significant. It is worth noting that brain weights were taken from males and females at the same age in the F_2 generation. Differences among dose groups were highly significant ($P \sim .003$) and interactions between group, dose and sex were not significant.

The pattern of distribution of means that emerges is very interesting, almost the mirror image of the pattern of distribution of means for the righting response, and the interpretation may well have a similar basis. In brain weight, the lowest mutagen dose produces the greatest increase in brain weight over that of controls followed by a slow more or less linear decrease in the brain size which however never falls to the level of controls. Our interpretation is that a modest increase in genetic variance induced by a relatively slight mutagen dose will permit the inbred experimental animal to develop a larger brain. With increasing amounts of variance, and the cumulative effects of deleterious mutants, these effects are masked increasingly with increasing mutagen dose. From examination of the results of the F_1 generation, where total amounts of mutagen induced variation would have been greater than in the F_2 generation (Table 6), it can be observed that the difference between the control and the lowest mutagen dose group is not as great as that between the same groups in the F_2 generation. The subsequent decrease in brain size with increasing dose falls to approximately the same level, relative to controls, as that in the F_2 generation.

It should be noted that there is the very exciting possibility that using these two traits, righting response and brain weight, the sensitivity of the test for very low mutagen doses is quite high.

With respect to variation in regard to brain weight variance, experimental variances in all six comparisons (two sexes, three doses), replicates combined, are lower than those of their appropriate controls, and two of these differences are significant.

Table 9. Variances of brain weight at about 13 1/2 weeks in F_2 generation progeny of three experimental and a single negative control group showing effects of TEM upon spermatozoa. Replicates are combined. Indications of statistical significance pertain to variance ratio comparisons with appropriate controls.

Dose (mg./kg.)	0	.075	.15	.30
Females	248.00	220.09	156.02*	198.53
Males	249.19	173.18	140.23*	184.96

* $P < .05$

Effects of Mutagen on Spermatogonia: F_1 Generation

As in the experiments of the first year there were very few strongly persuasive evidences of quantitative effects in F_1 progeny of mutagenized males where treatment was administered to spermatogonial stages. Brain weight stands out as an exception to this generalization. Dose was found to have a highly significant effect upon brain weight, with the progeny of mutagenized males in all except one of the sex-dose groups exhibiting greater brain weights than its appropriate control. An interesting difference in dose response between the sexes necessitates that dose comparisons be made separately for males and females.

With respect to tail length there were highly significant differences due to dose, but the pattern of mean distribution does not make a great deal of sense at this stage of analysis. There were also highly significant differences between groups, between sexes, and the dose by sex interaction was sufficiently great to approach significance. Analyses of this trait will continue in the future.

Analysis of the defecation portion of the open field test indicated that there were differences among dose groups of borderline significance, the general pattern of distribution suggesting an increase in mean value with increasing dose in a roughly linear fashion. These means, with sexes and replicates combined and adjusted for variation in litter size (which was not however significant), are presented in Table 10. Inasmuch as this trait was examined for the first time in the present year's experiments, it will be desirable to seek confirmation of this result in future experiments.

Differences due to dose with respect to hematocrit and body weight were not significant, although there were the expected highly significant differ-

ences between replicates and sexes. Variation in body weight variance in the first year's experiment showed a pattern of uniformly reduced variances among experimental groups relative to controls, and half of the six comparisons possible were significant reductions. In the results of the present year's experiment, this pattern is not seen. Of the twelve comparisons possible (two replicates, two sexes, three mutagen doses) only five experimental variances are lower than their appropriate controls.

Table 10. Means for brain weight at 13 1/2 weeks or more, tail length at seven weeks and defecation rate in F_1 generation progeny of three experimental groups and a single negative control for two experiments showing the effects of TEM upon spermatogonia.

Experiment	Dose (mg./kg.)	0	.075	.15	.30
II	Brain weight ♀	424.53	426.70	430.05	426.79
	♂	409.27	413.60	411.57	417.05
I	Tail Length	63.5	65.33	55.87	67.79
II		75.26	75.69	74.54	75.46
II	Defecation	3.28	3.54	3.80	3.83

Brain weight. Brain weight was not found to vary significantly with variation in litter size. However, there were highly significant differences ($P < .001$) between replicates and between sexes. There was also a significant dose x sex interaction ($P \sim .02$). Accordingly dose group comparisons were made within sexes. Examination of the general effect of dose suggests an increase in brain size that is more or less linear with increasing dose. The interpretation that has been suggested above for other brain weight effects would seem to apply equally well in this case. Variation in brain weight means is shown in Table 10 separately for males and females, but with replicates combined.

Tail length. As indicated above variation in tail length among dose groups was highly significant ($P < .001$), however the pattern that emerges is not so easily subject to interpretation. Since there are also highly significant differences ($P < .001$) between groups and sexes, comparisons must be made with considerable care. There does exist a parallel of some interest between the distribution of means in the present year and that of the first year's experiments, where the results were not cited because the pattern of distribution of means did not make much sense. Nevertheless, there was a significant difference among dose group means ($P \sim .02$). Means for the experiments of the first and second year, adjusted for litter size, are shown in Table 10 for comparison. In each experiment the mean associated with the middle mutagen dose is reduced

below the others, and that there tends to be an increase from control to the lowest mutagen dose followed by a significant drop at the middle mutagen dose and a subsequent increase to a maximum for the high mutagen dose. This unusual distribution is further supported by the observation that each of the replicates showed a parallel distribution of means.

Defecation. Distribution of means for the defecation portion of the open field test is provided for interest in Table 10. While the difference between dose groups only approaches significance ($P \sim .06$), the pattern that emerges is of interest. Differences between replicates and sexes approached significance ($P \sim .10$), but there were no significant interactions between dose, replicate, and sex, nor was there significant effect of litter size variation. The pattern that is evident is one of a more or less linear increase in defecation rate with increasing mutagen dose.

Effect of Mutagen on Spermatogonia: F_2 Generation

Again in contrast with the results of the first year where several traits exhibited interesting mean distributions associated with significant differences, in the present year only one trait, brain weight, showed strong evidence of potential usefulness. There was a highly significant difference among dose groups ($P < .01$), and the pattern of mean distribution paralleled that found in the F_2 generation of spermatozoa treatment groups. Analysis of data on hematocrit indicated that there were significant effects of dose, with means for experimental groups generally being lower than the control.

The analyses of data with respect to time of development of righting response, the defecation portion of the open field test, body weight at weaning and tail length at seven weeks failed to show either significant differences due to dose or variance distributions that would be of interest. With respect to body weight, but for males only, there was the predicted increase in variance for all experimental groups relative to appropriate controls, but this pattern, which had been evident for females also in the first year's experiment, was not extended to females in the present year's experiment.

Table 11. Means for brain weight at about 13 1/2 weeks and hematocrit in F_2 generation progeny of three experimental groups and a single negative control for two experiments showing the effects of TEM upon spermatogonia.

Experiment	Dose (mg./kg.)	0	.075	.15	.30
II	Brain weight C	419.27	414.95	416.35	418.67
	D	416.70	424.24	421.09	423.66
II	Hematocrit	46.53	46.23	45.96	46.40

Brain weight. Analysis of variation in brain weight revealed that there was a highly significant association with litter size ($P < .001$), and highly significant differences between replicates ($P \sim .003$) and sexes ($P < .001$). Differences among groups were also highly significant ($P \sim .006$), but a highly significant replicate x dose interaction necessitated that comparisons be carried out separately for each replicate. Experimental groups in replicate D exhibited brain sizes significantly greater than the control. However, differences among means in replicate C were not significant (Table 11).

Hematocrit. The analysis of results for variation in hematocrit indicated that there was not significant association with litter size, although there were highly significant differences ($P < .001$) between replicates and sexes. There were also significant differences ($P < .05$) due to dose. The pattern of mean distribution is in striking contrast with that of the first year's experiment which was not, however, associated with statistically significant differences. In the present year's experiment, the three experimental group means show a reduction in hematocrit below that of control, with the middle mutagen dose group showing the greatest depression relative to control. Dose means adjusted for litter size, with sexes and replicates combined, are shown in Table 11.

Discussion. Negative results observed in the first year with respect to effects of mutagenic agent on sex ratio were found again in the present year. As we reported in the results of the first year, reduced numbers of young carried to weaning by female progeny of mutagenized males, which had been a good indicator of mutagenic damage in X-ray studies, proved not to be so in our TEM studies. Similarly, in the second year's results there were no evidences to support such an effect.

While the general usefulness of body weight as a trait appeared to be much diminished in the results of the present study, especially the variance patterns which had appeared quite exciting in last year's study and in earlier work, effects on body weight means resulting from mutagenized spermatozoa still appear to be of usefulness. However, the very interesting effects associated with the F_1 and F_2 generation of mutagenized spermatogonia were not substantiated in the present year's study. The results for tail length parallel the body weight conclusions as they did in the prior year's study. Apparently usefulness of tail length is much diminished, but for mutagenized spermatozoa, especially in the F_1 generation, increases in tail length do appear to be indicative of mutagenic damage. Similarly decreases in variance both with respect to body weight and tail length in the F_1 generation appear to be indicative of mutagenic damage.

In contrast to the reduced usefulness of body weight and tail length, the time of development of the righting response appeared to be particularly useful as a diagnostic trait. The results of the present year's experiment are supported, in retrospect, by the results of the past year's experiment which were not in themselves significant, but which did parallel the results found in the present year. This mutagenic effect may be summarized as one of decrease or unaffected developmental time with very low mutagen dose, followed by increasing developmental times, i.e. reduced developmental rates, with increasing mutagen dose. This same pattern was found for F_1 and F_2 generation groups alike, with the F_2 generation low mutagen dose response being particularly interesting as a sensitive indicator of mutagenic damage.

All of the above traits have the prognosis for being useful and also have

the advantage of being relatively easily obtained. By contrast, brain weight at 13 1/2 or more weeks of age, a trait tried for the first time in the present year's experiments, proved to be very broadly useful, having considerable potential as an indicator of mutagenic damage for spermatogonia and spermatozoa alike and for F_1 as well as F_2 generation groups.

With respect to plans for the coming year, it is proposed employing the same design employed in the present year and including all of the traits employed in the present year, that we examine the effects of TEM on two additional strains of inbred mice, BALB/cby and C3H/HeJ, each in comparison with strain DBA/2J, the strain upon which our studies have been conducted to date. It is also our plan to eliminate one of the dose groups in order to increase numbers per group as much as is possible. The current plan is to employ mutagen doses of 0, .1 and .2 milligrams of TEM per kilogram of body weight.

We plan to administer the antibiotic terramycin uniformly to all females rearing young, control and experimental, F_1 and F_2 generation of all three strains, nine days after the birth of the litter.

During the past year we investigated the possibility of employing a trait, variation in red blood cell volume, which had been proposed in a research conference earlier. It was found that the equipment necessary to accumulate satisfactory quantities of data with respect to red blood cell volume was rather expensive, approximating $25,000.00 for the Coulter Counter and associated computer. At that time, we could not seriously consider the possibility of obtaining the equipment in the short time available. At this point, we have made arrangements with the Coulter Company to use a demonstration Counter for a limited period during which it is expected that we could acquire sufficient data to determine the potential usefulness of the trait. Should the trait prove to be useful, we would make efforts to obtain equipment for future studies.

It is our feeling that results of the first two years provide strong support for the continued development of a mutagenic assay based upon the use of quantitative traits in the detection of polygenic mutation damage such as would be expected to result from a wide variety of mutagenic agents. In particular, we feel that body weight, hematocrit level, righting response and brain size have demonstrated great potential as indicators of mutagenic effects. Others, including tail length, the defecation portion of the open field test, survival of young to weaning and sex ratio may also prove to be of some usefulness in detecting certain kinds of genetic damage.

Finally, we have developed in these two experiments substantial evidence that a late dominant lethal effect, originally observed by Soares (1972) produces a significant number of deaths at parturition. These deaths cannot be accounted for either by covariation with litter size or by upper estimates of misclassified "dead" uterine scars.

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Respectfully submitted,

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G 32-621

REPORT OF PROGRESS FOR RESEARCH CONTRACT NIEHS NO. NO1-ES-5-2135

June 25, 1977 through December 24, 1977

The experiments proposed for the third year were devoted to exploration of the effect of strain of experimental animal (mouse) on the genetic system, particularly with respect to quantitative traits. Traits proposed for study were those that appeared to be promising based upon the results of the experiments of the first and second years.

In accord with discussions held with the Project Officer, Dr. Eugene Soares, it was agreed that triethylene melamine (TEM) would continue to be employed as the mutagenic agent as in the experiments of the first and second year. In order to compensate for the effects of increased numbers of groups required to compare strains, doses were modified somewhat from previous experiments. It was determined that only two doses would be employed in addition to the control; these were .1 and .2 mg TEM per kilogram of body weight. In previous years doses of .075, .15 and .30 mg per kilogram of body weight had been used. Sample sizes were altered somewhat in order to compensate for greater losses due to dominant lethality with increasing mutagen dose. As in the experiment of the second year, the germ cell stages tested included only spermatozoa and spermatogonia.

Experiments of the first year had indicated that some traits did not appear to be particularly useful. One of these was time of eruption of incisors and this has been eliminated from subsequent experiments. During the second year, completion of the analysis of the serum cholesterol level data indicated that this also should be discarded. Of two new traits analyzed for the first time in the second year, one, brain weight at 13 1/2 or more weeks of age, appeared to be very useful, and the other, number of fecal pellets deposited in a five minute period, proved to be of borderline usefulness, but is rather easily taken. Accordingly it is planned that these two in addition to the six traits remaining from the experiments of the first year will be continued. These include: 1) young per female carried to weaning, 2) age of development of the righting response, 3) body weight at weaning, 4) length of tail at seven weeks of age, 5) sex ratio, 6) hematocrit at seven weeks, 7) number of fecal pellets deposited in five minutes, and 8) brain weight at 13 1/2 or more weeks of age.

It was planned that two strains of mice would be compared with the performance of the strain employed in previous experiments, DBA/2J. The strains selected were C3H/HeJ and BALB/cby. These two strains were selected primarily because of their distant relationship to DBA/2J strain and because of their acceptable productivity records.

It appears that in the experiments of each year at least one significant problem developed to prevent the achievement of anticipated numbers of progeny animals which are so important to the determination of effect in quantitative genetic studies. In the present year a faulty light switch, one which failed to turn off lights to produce a twelve-hour-on and twelve-hour-off daily cycle, was not discovered until it had already led to seriously

decreased productivity of both replicates of matings involving spermatozoan treated males. In fact, it was the significantly reduced productivity of our DBA/2J and C3H/HeJ crosses that led to the discovery of the defective equipment. Interestingly, the BALB/cby crosses were reasonably productive in spite of their constantly lighted environment. These mutagenized males will be retained for crosses involving spermatogonial treatment. However, it is also planned that the spermatozoan experiments will be repeated in the spring with a separate set of mice which have already been ordered from the Jackson Laboratory. Precise timing of this will depend upon the time at which these animals become available, and the complete analysis of data may not be completed until early in the final contract year. However, it is felt that it will be of great importance to acquire this information for the strains in the present contract year. There has been none of the illness in the present year that plagued our experiments in the first two years. As of the present date, the parental crosses for both replicates of the spermatozoan and spermatogonial treated germ cell stages have been carried out.

Design. In the experiments underway, parental females serving as parents for determination of the effects of different germ cell stages were assigned to temporally separated replicates A and B, which were mated to males which had been exposed to mutagenic treatment immediately prior to mating, thus involving mutagenized spermatozoa. Replicates C and D represent groups of females which were mated to males which had been exposed to mutagenic treatment over eight weeks prior to establishment of matings, thus the germ cells involved were mutagenized as spermatogonia. According to original plan, a total of 116 male mice of each strain were treated at about 11 weeks of age and mated immediately with 226 females for a period of one week. These matings were carried out in two separate replicates, A and B, involving equal numbers of females, and were conducted two weeks apart. After separation of the A and B matings, males were housed with females not involved in the experiment and remained so until spermatogonial stage matings are established. Males which had been involved in the group A matings were each mated with three replicate C females for a period of two weeks. Similarly males which had been involved with group B matings were each mated with replicate D female trios for a period of two weeks.

Methods. Parental males and females of replicate A were received from the Jackson Laboratory at ten weeks of age on October 31, 1977, as ordered. Males and females were housed five to a box, sexes separate until matings were established two weeks later. Triethylene melamine was administered by intraperitoneal injections in Hank's Balanced Salt Solution prepared immediately prior to injection. As in the previous experiments, a series of male mice were weighed to determine body weight, and all males within a dose group received the same quantity of carrier and TEM irrespective of individual variation in body weight. All mice received .25 cc of solution by injection, controls receiving the same quantity of carrier only. Injections were administered to replicate A males on November 14, 1977, and matings were established, two females to each male, two days after administration of injections.

Replicate B males and females were received from the Jackson Laboratory on November 15, 1977. Males were treated on November 28, 1977, and matings established two days later. This replicate was handled in precisely the

same manner as the first. All males treated survived for a period of 24 hours and until the completion of the portions of the experiment involving them.

For purposes of recognition, males of each strain of replicates A and B were numbered respectively 115 through 172 and 415 through 475. Females were similarly ear punched for identification and were numbered for groups A and B of each strain respectively 1 through 114 and 301 through 414.

As indicated earlier, the numbers of pairs in each of the treatment groups were adjusted to compensate for the reductions in number of young expected for the higher dose rates. Employing the results of the first year, it was determined that the total number of males assigned respectively to the control, 0.1 and 0.2 mg/kg groups would be 24, 30 and 60. Males to be assigned to each of these groups were selected at random, and equal numbers of males were included in A and B replicates. Similarly, females were assigned randomly to the different treatment groups in appropriate numbers. Following establishment of replicate A and B crosses, cages were labeled in such a way as to preclude group identification.

Replicate A matings were established on November 16, 1977; males were removed on November 23, 1977, and placed with females not involved in the experiment in an effort to maintain a normal level of sexual behavioral activity in the males. Approximately ten days later, in expectation of production of litters, females were separated, one to a box. Replicate B matings were established on November 30, 1977; males were removed on December 7, 1977. Approximately ten days later, females were separated, one to a box.

Females which were to be involved in matings to test the effects of treated spermatogonia were also handled in two replicates of 113 females of each strain in each replicate. The same males that had been involved in replicate A of the spermatozoa testing matings were assigned to replicate C in the spermatogonial matings. Similarly, males which had been involved in replicate B spermatozoan matings were assigned to replicate D of the spermatogonial matings. As described above, because of the small number of males involved in the control and lowest TEM dose groups of the spermatozoa test matings, it was necessary to mate three females to each male.

Females of replicate C were received from the Jackson Laboratory on December 20, 1977, at 10 weeks of age and numbered 601 to 714 by ear punch and housed five to a box until matings were established. Females were paired with males on January 10, 1978 for a two week mating period and were separated on January 24, 1978. Using identical procedures, females involved in the replicate D matings were received from the Jackson Laboratory on January 4, 1978 and numbered 901 to 1014 by ear punch. Replicate D matings were set on January 24, 1978 and were terminated two weeks later on February 7, 1978.

Each of the three treatment groups in each strain was represented by twelve males in each of the replicates, C and D, for a total of 216 males. The 339 females of each of the two replicates were assigned randomly to males of their own strain of the different groups, three females per male. Following termination of the final matings, males of the parental generation were removed, and females were isolated, one to a box, in anticipation of the production of litters.

As F_1 young were produced, they were assigned temporary numbers by toe clipping within litters. Using this identification, offspring were tested for the ability to right (righting response). Subsequently, the young were weaned at four weeks of age, a permanent number was assigned, and the animals were identified by coded ear punching. Sex was recorded at birth and again at weaning (four weeks) along with body weight. The fecal count portion of the open field test was done at five weeks, and at approximately seven weeks the tail length was recorded and the hematocrit determined.

A summary of litter production in Replicates A and B is given in Table 1. Strain DBA/2J appears to be the worst producer, particularly in higher TEM dose groups. Strain C3H/HeJ is better than DBA/2J because of a lower rate of litter destruction. Strain BLAB/cby is a strong producer due to a higher fertility rate and a lower rate of litter destruction than in strains DBA/2J and C3H/HeJ. In strain DBA/2J, a total of 46 litters were produced based upon observation at birth. Of these litters 20 (43.5%) had some young which survived to weaning. In comparison, strain C3H/HeJ produced a total of 45 litters at birth of which 37 (82.2%) had some young surviving to weaning, and strain BALB/cby produced 96 litters at birth of which 90 (93.8%) had some young which survived to weaning. Although total production was somewhat low, strains C3H/HeJ and BALB/cby produced an adequate number of young in each of the three dose groups. Strain DBA/2J however, had very poor production in the highest dose group (.2 mg. TEM/kg.).

Autopsy of the replicate A and B parental females has been completed and preliminary results of the uterine examinations will be given in the next section. Replicate C matings have just finished producing litters, and replicate D matings are just beginning to produce litters. Data from F_1 young in replicates A and B have been recorded for righting response, tail length, body weight at weaning, the fecal portion of the open field test, and hematocrit. In replicate C, data are being obtained on the righting response of the F_1 progeny.

Results. Compilations of the data on dominant lethality and related losses for the A and B replicates for the three inbred strains examined are shown in Tables 2, 3, and 4 respectively for strains DBA/2J, BALB/cby, and C3H/HeJ. While these data have not yet been submitted to statistical analysis, it is evident that the trends shown parallel the results of the experiments of the first and second years carried out for DBA/2J strain. For both replicates of the BALB/cby a decrease in the number of live scars with increasing dose of TEM is clearly indicated. The same effect, on the average, obtains with strain DBA/2J although average replicate performance is not precisely reflected in within replicate trends. For example, in replicate A, the .10 mg. TEM/kg. dose group showed slightly higher live scar counts than the control group. The difference, however, is not likely significant. The situation with respect to the C3H/HeJ strain is interesting in that the number of live scars increases in both replicates in the .10 mg. TEM/kg. dose group over controls, and the differences are appreciable. A drop in live scars per female in the .20 mg. TEM/kg. dose group to below the control means is of even greater magnitude.

The three strains show parallel trends with respect to total live births per pregnant female. In each replicate of all three strains there is a consistent decrease in number of live births with increasing TEM dose. The same trend is evident with respect to total number of young weaned per female weaning young with the exception that for the C3H/HeJ strain there is essentially no difference between total young weaned per female for the .10 and .20 mg. TEM/kg. dose groups.

Table 1. Litter sizes at birth and at weaning for the three mouse strains employed as experimental animals with the three doses of TEM. Results from Replicates A and B (parental animals mated for seven days immediately following male treatment) were combined.

DBA	Litter size	0	1	2	3	4	5	6	7	8	9	10	11	12	13
	birth	35	1	3	6	–	3	4	2	1					
0															
	wean	42	–	–	3	1	3	3	2	1					
	birth	50	2	3	7	2	2	–	1						
.1															
	wean	61	–	2	1	2	1	1							
	birth	112	1	5	4										
.2															
	wean	121	–	1	–										
C3H	birth	31	–	1	–	2	2	2	–	1	1	1			
0															
	wean	31	–	1	–	2	2	2	–	1	1	1			
	birth	44	2	1	2	3	5	3	2	1					
.1															
	wean	49	–	2	1	4	4	2	–	1					
	birth	106	2	2	3	3	4	2							
.2															
	wean	110		1	2	3	4	2							
BALB	birth	3	–	–	1	3	3	3	5	3	6	1	3	–	1
0															
	wean	3	–	–	1	4	4	5	4	1	6	1	3	–	1
	birth	32	–	7	3	3	7	5	3	1					
.1															
	wean	32	–	7	2	2	8	4	3	1					
	birth	89	9	11	5	2	1	2							
.2															
	wean	92	7	10	6	1	2	1							

Table 2. Dominant lethality parameters with standard errors and sample sizes of Replicate A and B series (DBA/2J strain) females and their F_1 young. Females belonging to the two experimental and a single negative control group were mated for a period of seven days immediately following treatment, thus represent effects of mutagenic agent upon spermatozoa.

	TEM dose, mg./kg. of body weight		
	0	.10	.20
Replicate A			
Live Scars/♀	2.09 ± .49(31)	2.20 ± .41(35)	.61 ± .16(61)
Dead Scars/ ♀	.13 ± .06(31)	.60 ± .15(35)	.20 ± .07(61)
Total Scars/ ♀	2.23 ± .52(31)	2.80 ± .49(35)	.80 ± .18(61)
Total Live Births/ ♀	3.07 ± .63(15)	2.25 ± .49(20)	.95 ± .30(19)
Total Weaned/ ♀	4.86 ± .67(7)	3.60 ± .74(5)	2(1)
Percent Dead Scars of Total	5.8	21.4	24.5
Percent Perinatal Losses	29.2	44.1	51.4
Replicate B			
Live Scars/ ♀	2.29 ± .65(24)	.47 ± .19(32)	.38 ± .12(61)
Dead Scars/ ♀	.29 ± .11(24)	.13 ± .07(32)	.07 ± .03(61)
Total Scars/ ♀	2.58 ± .70(24)	.59 ± .21(32)	.44 ± .14(61)
Total Live Births/ ♀	4.00 ± .84(10)	1.57 ± .48(7)	.45 ± .25(11)
Total Weaned/ ♀	5.67 ± .61(6)	3(1)	0
Percent Dead Scars of Total	11.3	21.1	14.8
Percent Perinatal Losses	27.3	26.7	78.3

Table 3. Dominant lethality parameters with standard errors and sample sizes of Replicate A and B series BALB/cby females and their F_1 young. Females belonging to the two experimental and a single negative control group were mated for a period of seven days immediately following treatment, thus represent effects of mutagenic agent upon spermatozoa.

	TEM dose, mg./kg. of body weight		
	0	.10	.20
Replicate A			
Live Scars/ ♀	8.19 ± .84(21)	3.50 ± .63(32)	1.37 ± .29(59)
Dead Scars/ ♀	.19 ± .09(21)	.69 ± .25(32)	.71 ± .19(59)
Total Scars/ ♀	8.38 ± .84(21)	4.19 ± .68(32)	2.08 ± .39(59)
Total Live Births/ ♀	6.74 ± .74(19)	3.75 ± .55(20)	1.38 ± .27(24)
Total Weaned/ ♀	6.94 ± .68(18)	4.29 ± .50(17)	2.14 ± .25(14)
Percent Dead Scars of Total	2.3	16.4	46.7
Percent Perinatal Losses	25.6	33.0	59.3
Replicate B			
Live Scars/ ♀	8.71 ± .87(14)	2.69 ± .64(29)	1.27 ± .31(59)
Dead Scars/ ♀	.07 ± .07(14)	.62 ± .22(29)	.78 ± .16(59)
Total Scars/ ♀	8.78 ± .87(14)	3.31 ± .72(29)	2.05 ± .41(59)
Total Live Births/ ♀	7.46 ± .60(13)	3.86 ± .58(14)	1.58 ± .39(24)
Total Weaned/ ♀	7.46 ± .60(13)	4.42 ± .50(12)	2.69 ± .46(13)
Percent Dead Scars of Total	.8	18.8	38.0
Percent Perinatal Losses	20.5	30.8	50.7

Table 4. Dominant lethality parameters with standard errors and sample sizes of Replicate A and B series C3H/HeJ females and their F_1 young. Females belonging to the two experimental and a single negative control group were mated for a period of seven days immediately following treatment, thus represent effects of mutagenic agent upon spermatozoa.

	TEM dose, mg./kg. of body weight		
	0	.10	.20
Replicate A			
Live Scars/ ♀	1.86 ± .78(22)	2.65 ± .62(31)	1.62 ± .34(60)
Dead Scars/ ♀	.18 ± .18(22)	.03 ± .03(31)	.10 ± .06(60)
Total Scars/♀	2.05 ± .82(22)	2.68 ± .62(31)	1.72 ± .35(60)
Total Live Births/ ♀	6.60 ± 1.78(5)	3.75 ± .85(12)	1.74 ± .52(19)
Total Weaned/♀	8.25 ± .85(4)	5.20 ± .37(5)	4.43 ± .48(7)
Percent Dead Scars of Total	8.9	2.6	5.8
Percent Perinatal Losses	19.5	45.1	66.0
Replicate B			
Live Scars/♀	2.32 ± .76(19)	2.77 ± .62(31)	2.06 ± .37(64)
Dead Scars/♀	.11 ± .11(19)	.16 ± .08(31)	.05 ± .03(64)
Total Scars/♀	2.42 ± .78(19)	2.94 ± .66(31)	2.11 ± .38(64)
Total Live Births/♀	3.71 ± .78(7)	2.92 ± .69(13)	1.42 ± .40(24)
Total Weaned/♀	4.33 ± .56(6)	4.13 ± .69(8)	4.14 ± .40(7)
Percent Dead Scars of Total	4.3	5.5	2.2
Percent Perinatal Losses	40.9	55.8	74.2

Of particular interest is variation in percent dead scars of total scars and percent perinatal losses, which has been defined as: live scars - live young observed at birth/live scars. With respect to percent dead scars of total, the BALB/cby strain shows a classical increase from a very low control value of a few percent to a value somewhere between 1/3 and 1/2. Data for strain DBA/2J also shows an increase from a control value (which is however somewhat higher than the control value for BALB/cby) to a figure somewhat less than 1/4. As in experiments of previous years the difference between high and low TEM dose groups in percent dead scars of total is not as great as that between control and the low TEM dose group. Our data for strain C3H/HeJ are particularly interesting in that they suggest that the classical dominant lethal test would be of very little usefulness in determining the effects of mutagenic action. There is, in effect, no difference between treated groups and control with respect to percent dead scars of total. With respect to percent perinatal losses, however, there is a clear and consistent increase with increasing TEM dose from a very high control value to values two or three times as great as control for the .20 mg. TEM/kg. dose group. Perinatal losses were equally clearly shown to increase for the BALB/cby strain and the DBA/2J strains.

With respect to total scars, as has been discussed in earlier reports, it is generally expected that slight decreases, if any, for lower mutagen doses will be followed by more dramatic decreases at higher doses. The data from the results of experiments of previous years with strain DBA/2J were consistent with this expectation as are the current results with strain DBA/2J. Such reductions in scar numbers at higher mutagen doses are interpreted as resulting from preimplantation losses, which would be expected to increase at high mutagenic exposures. Results with respect to strain BALB/cby are in interesting contrast in that preimplantation losses are appreciable at the lowest TEM dose and increase again appreciably at the highest dose. By contrast, total scars actually seem to increase for strain C3H/HeJ followed by an appreciable decrease at the highest mutagen dose.

As reported in our last Annual Report (for the period December 25, 1976 through June 22, 1977) the experiments of the first two years resulted in the development of substantial evidence that a late dominant lethal effect produces a significant number of deaths at parturition. In a collaborative effort based upon data compiled independently by Dr. J. Favor and Dr. E. Soares, a manuscript has been prepared and submitted for publication to Mutation Research that demonstrates that these late dominant lethal, or perinatal losses as we have termed them, cannot be accounted for either by covariation with litter size or by upper estimates of misclassified "dead" uterine scars. Data from the experiments of the present year offer additional confirmation of this effect.

Also as reported in the cited Annual Report, we investigated the possibility of employing a trait, variation in red blood cell volume, which had been proposed in a research conference earlier. The equipment required to produce satisfactory quantities of data with respect to red blood cell volume would require an outlay of approximately $25,000. We reported that an arrangement had been made with the Coulter Company to use a demonstration piece of equipment for a limited period to determine if we could acquire sufficient data to determine the potential usefulness of the trait. At the present time the Coulter Counter and associated computer are on loan in our laboratory, and we hope to be able to run a sufficient number of samples to obtain a rough idea as to the potential usefulness of this trait.

As indicated above, the productivity of spermatozoan stage crosses particularly for strains DBA/2J and C3H/HeJ were significantly reduced, probably as the result of a faulty light switch, to such an extent that it was judged desirable to attempt to repeat these crosses in the present year. Inasmuch as the light switch was repaired prior to spermatogonial stage crosses, the originally treated males have been employed as planned in the matings and no repetition of these matings is planned. However, we have ordered numbers of animals of all three strains sufficient to repeat the spermatozoan stage matings with the expectation of accumulating data from their F_1 progeny and from an F_2 generation. In view of the significant delay in repeating this experiment, it is likely that data for the F_2 generation will not be available for analysis at the time of the December 24, 1978 Annual Report preparation. Any such analyses will be carried over and provided in the report for the period ending December 24, 1978.

There is an additional advantage to be gained by the repetition of spermatozoan stage crosses. At the present moment, the BALB/cby strain would appear to be a strain of choice, other things remaining the same, for the kinds of experiments being conducted. However, we do not yet have data on the reaction of the BALB/cby strain to effects upon quantitative traits. It will be of considerable importance, therefore,to have two replicates of BALB/cby strain spermatozoan cross results to determine the usefulness of the strain with respect to quantitative traits. (It will be recalled that this was the only strain to produce sufficient numbers of spermatozoan stage progeny to justify setting up F_1 matings). Should our analyses be positive, we would propose that the BALB/cby strain be used in the experiments of the final year in place of the DBA/2J strain for purposes of comparison with two new strains to be tested as proposed in the original research contract proposal.

Respectfully submitted,

John W. Crenshaw, Jr.

John W. Crenshaw, Jr.
Project Director

G-32-621

REPORT OF PROGRESS FOR RESEARCH CONTRACT NIEHS NO. NO1-ES-5-2135

December 25, 1977 through June 25, 1978

This report includes preliminary analysis of data acquired to date in the experiment of the third year of the referenced research contract, and involved an exploration of the effect of strain of experimental animal inbred (mouse) on the effect of mutagenic agent in affecting the expression of quantitative traits. As in earlier experiments, triethylene melamine (TEM) was the mutagenic agent employed. In order to maximize the number of animals in each group only two doses (.1 and .2 mg TEM per kilogram of body weight) were employed in addition to a control group, instead of the three doses (.075, .15 and .30 mg per kilogram of body weight) which had been employed in prior experiments. The number of matings set in each dose group was adjusted to compensate for dominant lethal effects of treatment in the high dose groups in such a way that similar numbers of F_1 progeny would be produced in all groups. TEM treatment effects were tested on spermatozoa and spermatogonial stages of spermatogenesis.

The quantitative traits analyzed in the experiment reported here to determine the effect of mutagenic treatment on F_1 and F_2 progeny of treated males included 1) age of development of the righting response, 2) body weight at weaning, 3) the defecation portion of the open field test, 4) tail length at seven weeks of age, 5) hematocrit at seven weeks of age, and 6) brain weight at 13 1/2 or more weeks of age. In addition to these quantitative traits, productivity traits were also investigated to determine if treatment was effective. These traits included 1) per cent dead implantation scars of total scars, 2) total implantation scars, 3) per cent females born, 4) total born, 5) per cent females weaned and 6) total weaned. As in prior experiments, the productivity traits 1) and 2), determined by inspection of uterine scars in parental generation females, were collected for F_1 generation young only while the remaining productivity traits, 3) through 6), were collected in both F_1 and F_2 generations.

The major thrust of the experiment reported here involved a comparison of the performance of the strain employed in previous experiments, DBA/2J, with two untested strains, C3H/HeJ and BALB/cByJ. These strains were selected for this experiment because of their distant relationship to DBA/2J and because of their acceptable productivity records. As indicated in the Report of Progress for the period ending December 24, 1977, relatively low productivity was realized for the strains DBA/2J and C3H/HeJ which led to the discovery of defective light timing equipment. As will be seen below, correction of the faulty light switch did not improve the productivity of these strains significantly in subsequent crosses, suggesting that other factors (e.g. time of year, intrinsic changes in strain or unsuspected disease) may have also been of importance. By contrast with the two above strains, the BALB/cByJ crosses were highly productive. Realizing that the low productivity of the first two strains would leave our facilities relatively

uncrowded later in the year, a decision was made to attempt a second replicate of BALB/cByJ to repeat the experiments at least for F_1 young, to whatever extent might be possible in the present year. This decision, which has been effected in crosses now well under way, turned out to be particularly advantageous inasmuch as discussions between the principal investigator and contract officer have resulted in a decision to devote total efforts to the testing of strain BALB/cByJ in the final contract year. Thus, all that can be learned at the present time with respect to the response of this strain will be particularly useful in the final year.

Design of Experiment. In the experiments of the past year, female parents employed to determine the effects of spermatozoa treated at different germ cell stages were assigned to one of four replicates. Replicates A and B, carried out two weeks apart, were made up of females mated to males which had been exposed to mutagenic treatment immediately prior to mating, thus involving mutagenized spermatozoa. These matings involved mated trios of two females with each male. Replicates C and D were temporally separated replicate groups of females which were mated (in a ratio of 3 ♀ :1 ♂) to males which had been exposed to mutagenic treatment over eight weeks prior to establishment of mating. Thus the germ cells involved were mutagenized as spermatogonial cells. In Replicates A and B, a total of 116 male mice of each of the three strains tested were treated at about 11 weeks of age and mated immediately with 226 females of each strain for a period of one week. These matings were divided into two replicates involving equal numbers of females as described above.

After separation of the matings in Replicates A and B, males were housed with females not involved in the experiment until spermatogonial stage matings had been established. Males which had been involved in the Replicate A matings were each mated with three Replicate C females for a period of two weeks. A total of 114 males and 342 females, evenly divided among the three strains, were involved in these matings. Two weeks later Replicate D matings were similarly established involving the same males that had been parents in the Replicate B crosses and were maintained for two weeks. Again 114 males and 342 females, evenly divided among the three strains, were involved.

Females were isolated from each other 10 days after the initiation of matings and checked daily for the presence of a litter through day 21 after the termination of matings. Young were sexed and marked by toe clipping for identification at birth. Starting on day 5, progeny were checked for the ability to right (righting response) and were checked daily until the trait appeared. All animals were weaned at four weeks of age, at which time body weight and sex were recorded and individuals were permanently marked by coded ear punching. The defecation portion of the open field test was conducted on mice at 5 weeks of age while tail length and hematocrit measures were taken on mice at approximately 7 weeks of age.

Matings of F_1 individuals to produce F_2 progeny were established at between 11 and 12 weeks of age. Pair crosses (1♀ :1♂) were established between individuals taken randomly, except that sibling crosses were precluded, within a dose, a replicate, and a strain. Two replicates, A and

B, were produced by the F_1 young in the spermatozoan treatment group, the progeny respectively of Replicates A and B of parental crosses. A total of 116 Replicate A pairs and 111 Replicate B pairs were established, employing BALB/cByJ strain animals only. It was decided that there were too few F_1 animals in the other strains to produce meaningful results. Facilities were strained by the establishment of Replicate C F_1 crosses to the extent that Replicate D F_1 crosses could not be set. Accordingly, the prospective Replicate D F_1 parents were held pending the availability of sufficient cages to house them. A total of 336 BALB/cByE pairs, 40 C3H/HeJ pairs, and 35 DBA/2J pairs were established in Replicate C F_1 crosses. These figures reflect the relative productivity of the different strains quite accurately. All F_1 matings were single pair crosses and were maintained for a period of two weeks. Males were separated from females of the F_1 matings and brain weights were recorded as early as possible after about 13 1/2 weeks. For females, brain weights were recorded when litters of the group had been weaned.

As indicated above the relatively high productivity of BALB/cByJ strain as contrasted with the relatively low productivity of the other two strains led to a decision to repeat Experiment III for the BALB/cByJ strain only. Accordingly, Experiment IV Replicates A and B, involving a total of 119 males and 238 females, approximately evenly divided between the two replicates, were established in a precise repetition of Experiment III, except that the effects of mutagenic action on spermatozoa only were tested. Effects of spermatogonia were not investigated. Thus, there were no Experiment IV Replicates C and D. Table 1 details the scheduling of parental and F_1 generation matings.

Table 1. Schedule of matings

A. Parental generation

	Replicate					
	IIIA	IIIB	IVA	IVB	IIIC	IIID
Mice received	10/31/77	11/15/77	5/9/78	5/23/78	12/20/77	1/4/78
Matings	11/16	11/30	5/25	6/8	1/10	1/24
Matings separated	11/23	12/7	6/1	6/15	1/24	2/7

B. F_1 generation crosses

	Replicate		
	IIIA	IIIB	IIIC
Matings set	2/24/78	3/13/78	5/1/78-5/3/78
Matings separated	3/10	3/27	5/15 - 5/17

The mutagenic treatment itself consisted of intraperitoneal injection of triethylene melamine carried in .25 cc Hank's balanced salt solution prepared immediately prior to injection. Control males received the same quantity of carrier only. Doses were based on the mean body weight of a random sample of the mice to be treated. The numbers of matings set in each dose were adjusted to compensate for dominant lethal effects in the higher doses so as to produce similar numbers of F_1 progeny. The total number of males treated in Replicates A and B combined, in the dose groups, 0.0, .1 and .2 mg/kg of body weight were respectively 24, 30 and 60. All animals were identified by a coded number so that observations were blind with respect to dose. Since dominant lethal effects in spermatogonial treatment groups are very slight, if present, Replicates C and D dose groups were established with approximately equal numbers of matings. With unequal numbers of males in the dose groups established for Replicates A and B, the number of males mated in each dose group of Replicate C and D was equal to the number of males in the control dose group, which had the smallest number of males involved. In order to mate all females each male had to service three females. Thus, quartets in the ratio of 3 :1 were established for Replicates C and D.

As of the present moment data are available for Replicates A and B parental crosses with respect to productivity and quantitative traits, except that brain weights for a few males which were involved in translocation studies will not be included. With respect to Replicates C and D, data are available on quantitative traits except for brain weights in Replicate D and females still weaning young in Replicate C. For F_2 progeny of F_1 crosses in Replicates A and B data on all quantitative traits except brain weight are available. F_2 young of Replicate C F_1 crosses have just been weaned and Replicate D matings have not been established. Accordingly, quantitative traits will not be discussed for these two groups. Similarly, Experiment IV Replicates A and B parental crosses have respectively just had litters or are ready to have litters and the data available would not be meaningful. Accordingly discussions under results will be limited to those for which available data are reasonably complete, Replicates A and B, parental crosses only.

Results: Dominant Lethality. Data on dominant lethality and related losses for the A and B replicates of all three inbred strains were discussed in the Report of Progress for the period ending December 24, 1977. Tables 2, 3, and 4, respectively for strains DBA/2J, BALB/cByJ, and C3H/HeJ, are taken from that document and included in the present for completeness. As pointed out earlier, the results parallel the results of experiments of the first and second years carried out with DBA/2J strain. With respect to strain comparisons, certain interesting differences are apparent. While total live scars tend to decrease with increasing dose for both DBA/2J and BALB/cByJ, strain C3H/H3J showed an increase in live scars between controls and the lowest mutagen dose followed by a drop to a level below that of controls for the highest mutagen dose. These trends were evident in both replicates. All three strains parallel one another closely with respect to live births per female which decreases uniformly in both replicates of all three strains with increasing dose. Similarly total young

weaned per female carrying young to weaning decreases with increasing dose in all three strains. However the C3H/HeJ strain shows very slight differences in one replicate. In the traditional measurement of dominant lethality, per cent dead scars of total scars, DBA/2J strain exhibited the expected trend of an increase with increasing dose, although there is some irregularity between replicates. By contrast, the BALB/cByJ strain exhibits a clear increase from a very low control value to a very high value with the highest mutagen dose supported by closely parallel and regular replicate responses. Strain C3H/HeJ is remarkable in that clear evidence of dominant lethality by the traditional measure is lacking. In both replicates the highest mutagen dose shows a face value decrease in per cent dead scars of total, but all differences between experimental groups and control are slight and show no evidence of a dominant lethal effect.

Finally, of particular interest is variation in perinatal dominant lethality. Investigators on this project (Crenshaw and Favor) in collaboration with Dr. Eugene Soares have recently determined that per cent perinatal losses, defined as live scars minus live young observed at birth/live scars, increase with increasing mutagen dose in experiments involving three strains of mice and three mutagenic agents. Results of the present study support this observation. In the DBA/2J strain there is a general increase in perinatal losses with increasing mutagen dose although, as might be expected, there is some variation between replicates within strain. In strain BALB/cByJ, also as might be expected, there is a clear increase in perinatal losses with increasing mutagenic dose in each replicate. The C3H/HeJ strain is interesting in that per cent perinatal losses increase in both replicates with increasing dose in striking contrast to the lack of a dominant lethal effect using the standard measure.

Detailed statistical analyses of the above results have not yet been carried out. The late start of these experiments, necessitated by a delay in arrival of the mice employed, has not left time for computer analysis. These analyses are being conducted, however, and significant results will be summarized in the next semi-annual report. Standard errors have been computed for those parameters for which such a computation is appropriate and do provide an indivation of significance. It might also be noted that the magnitude of the differences with respect to per cent dead scars of total and per cent perinatal losses would suggest clearly significant differences among both groups for BALB/cByJ strain and an approach to significant differences among groups for DBA/2J strain.

Quantitative Traits. The primary goal of the series of experiments conducted over the past three years has been to determine the possible usefulness of quantitative traits in mice as indicators of mutagenic activity. As reported earlier, in studies of the first year (and in earlier experiments) indications were that mutagen induced polygenic mutations, that is mutations of minor effects, do have an influence upon the means and the variances of some quantitative traits under some conditions. Some traits appeared to be of particular usefulness while others showed no effect of mutagenic activity. It was also found that effects due to mutagenic treatment could be detected in both the F_1 and F_2 generation progeny of males

Table 2. Dominant lethality parameters with standard errors and sample sizes of Replicate A and B series (DBA/2J strain) females and their F_1 young. Females belonging to the two experimental and a single negative control group were mated for a period of seven days immediately following treatment, thus represent effects of mutagenic agent upon spermatozoa.

	TEM dose, mg./kg. of body weight		
	0	.10	.20
Replicate A			
Live Scars/♀	2.09 ± .49(31)	2.20 ± .41(35)	.61 ± .16(61)
Dead Scars/ ♀	.13 ± .06(31)	.60 ± .15(35)	.20 ± .07(61)
Total Scars/ ♀	2.23 ± .52(31)	2.80 ± .49(35)	.80 ± .18(61)
Total Live Births/ ♀	3.07 ± .63(15)	2.25 ± .49(20)	.95 ± .30(19)
Total Weaned/ ♀	4.86 ± .67(7)	3.60 ± .74(5)	2(1)
Percent Dead Scars of Total	5.8	21.4	24.5
Percent Perinatal Losses	29.2	44.1	51.4
Replicate B			
Live Scars/ ♀	2.29 ± .65(24)	.47 ± .19(32)	.38 ± .12(61)
Dead Scars/ ♀	.29 ± .11(24)	.13 ± .07(32)	.07 ± .03(61)
Total Scars/ ♀	2.58 ± .70(24)	.59 ± .21(32)	.44 ± .14(61)
Total Live Births/ ♀	4.00 ± .84(10)	1.57 ± .48(7)	.45 ± .25(11)
Total Weaned/ ♀	5.67 ± .61(6)	3(1)	0
Percent Dead Scars of Total	11.3	21.1	14.8
Percent Perinatal Losses	27.3	26.7	78.3

Table 3. Dominant lethality parameters with standard errors and sample sizes of Replicate A and B series BALB/cby females and their F_1 young. Females belonging to the two experimental and a single negative control group were mated for a period of seven days immediately following treatment, thus represent effects of mutagenic agent upon spermatozoa.

	TEM dose, mg./kg. of body weight		
	0	.10	.20
Replicate A			
Live Scars/ ♀	8.19 ± .84(21)	3.50 ± .63(32)	1.37 ± .29(59)
Dead Scars/ ♀	.19 ± .09(21)	.69 ± .25(32)	.71 ± .19(59)
Total Scars/ ♀	8.38 ± .84(21)	4.19 ± .68(32)	2.08 ± .39(59)
Total Live Births/ ♀	6.74 ± .74(19)	3.75 ± .55(20)	1.38 ± .27(24)
Total Weaned/ ♀	6.94 ± .68(18)	4.29 ± .50(17)	2.14 ± .25(14)
Percent Dead Scars of Total	2.3	16.4	46.7
Percent Perinatal Losses	25.6	33.0	59.3
Replicate B			
Live Scars/ ♀	8.71 ± .87(14)	2.69 ± .64(29)	1.27 ± .31(59)
Dead Scars/ ♀	.07 ± .07(14)	.62 ± .22(29)	.78 ± .16(59)
Total Scars/ ♀	8.78 ± .87(14)	3.31 ± .72(29)	2.05 ± .41(59)
Total Live Births/ ♀	7.46 ± .60(13)	3.86 ± .58(14)	1.58 ± .39(24)
Total Weaned/ ♀	7.46 ± .60(13)	4.42 ± .50(12)	2.69 ± .46(13)
Percent Dead Scars of Total	.8	18.8	38.0
Percent Perinatal Losses	20.5	30.8	50.7

Table 4. Dominant lethality parameters with standard errors and sample sizes of Replicate A and B series C3H/HeJ females and their F_1 young. Females belonging to the two experimental and a single negative control group were mated for a period of seven days immediately following treatment, thus represent effects of mutagenic agent upon spermatozoa.

	TEM dose, mg./kg. of body weight		
	0	.10	.20
Replicate A			
Live Scars/ ♀	1.86 ± .78(22)	2.65 ± .62(31)	1.62 ± .34(60)
Dead Scars/ ♀	.18 ± .18(22)	.03 ± .03(31)	.10 ± .06(60)
Total Scars/♀	2.05 ± .82(22)	2.68 ± .62(31)	1.72 ± .35(60)
Total Live Births/ ♀	6.60 ± 1.78(5)	3.75 ± .85(12)	1.74 ± .52(19)
Total Weaned/♀	8.25 ± .85(4)	5.20 ± .37(5)	4.43 ± .48(7)
Percent Dead Scars of Total	8.9	2.6	5.8
Percent Perinatal Losses	19.5	45.1	66.0
Replicate B			
Live Scars/♀	2.32 ± .76(19)	2.77 ± .62(31)	2.06 ± .37(64)
Dead Scars/♀	.11 ± .11(19)	.16 ± .08(31)	.05 ± .03(64)
Total Scars/♀	2.42 ± .78(19)	2.94 ± .66(31)	2.11 ± .38(64)
Total Live Births/♀	3.71 ± .78(7)	2.92 ± .69(13)	1.42 ± .40(24)
Total Weaned/♀	4.33 ± .56(6)	4.13 ± .69(8)	4.14 ± .40(7)
Percent Dead Scars of Total	4.3	5.5	2.2
Percent Perinatal Losses	40.9	55.8	74.2

subjected to mutagenic treatment. Experiments completed the second year supported certain conclusions arrived at in the first year; other traits were found to be not so broadly useful as had been hoped. Some traits which had not appeared to be of particular usefulness in the first year's experiment showed greater promise in the second year. One trait not employed in the first year, brain weight, proved to be of particular interest in experiments of the second year.

While information from either generation may be of interest as an indicator of genetic damage resulting from mutagenic treatment, in general, effects observed in the F_2 generation are more certainly attributable to a genetic basis than those of the F_1 generation. Even so, genetic causation is an entirely reasonable hypothetical explanation of observed F_1 generation effects. However, the possibility of certain kinds of relayed toxic effects cannot be absolutely ruled out.

During the past year, an effort was made to examine the effects of mutagenic treatment on two new strains of inbred mice, employing the same experimental design that had been employed in earlier years with strain DBA/2J. As indicated above, these strains were BALB/cByJ and C_3H/HeJ. The necessity of obtaining reasonably large numbers of all three strains at the same time caused a significant delay in initiation of experiments, and data have been analyzed only for the effects of mutagenic agents on spermatozoa in the F_1 generation. These results are presented in the analyses to follow. Analyses of the effects of mutagen on the F_2 generation of spermatozoan treated germ cells and upon the F_1 and F_2 generations of spermatogonial treatments will be dealt with in subsequent reports.

Effects of Mutagen on Spermatozoa: F_1 Generation

The productivity of strain DBA/2J in experiments of the current year was considerably poorer than had been projected with the result that little usable data are available for comparison of treatment effects in F_1 generation animals. Since these animals will serve as parents for F_2 progeny, it is likely that little data will be available also for these comparisons. Hopefully, there will be more useful data available for spermatogonial treatment effects, particularly since dominant lethal effects will be of negligible importance in these matings.

Strain C_3H/HeJ, which is being employed for the first time in these experiments, also proved to be not entirely satisfactory in regard to its productivity, although some comparisons of interest did emerge. Inasmuch as modest numbers of individuals are available in each treatment group, it is anticipated that numbers available for analysis in the F_2 generation will be much more nearly satisfactory, as will numbers available in the spermatogonial treatment groups, F_1 and F_2 generation. The most exciting results of this year have hinged upon the very high productivity of strain BALB/cByJ. While there has not been time to subject these data to computer analysis, which will include the critical partitioning of the effects of litter size, the results to be presented below have been analyzed by ANOVA and by Student's

t-test where appropriate.

The most exciting results to emerge indicate that strain BALB/cByJ shows a linear increase in body weight with increasing mutagen dose. Differences among means were highly significant ($p < .01$) based upon a factorial ANOVA. This result is the same as was reported for strain DBA/2J in experiments of the first and second year. With respect to the time of development of the righting response, most exciting results were also found. With strain BALB/cByJ the time of development of the righting response increased with increasing mutagen dose in a roughly linear manner, and the differences proved to be significant by factorial ANOVA. This same result was also found in the experiments of the second year (but not those of the first) for strain DBA/2J. This same pattern can be visualized in the experiments of the first year with DBA/2J, but the differences among groups were not significant.

An interesting pattern was observed with respect to the defecation portion of the open field test in strain BALB/cByJ which, however, proved to be not significant based upon factorial ANOVA. The pattern involved a consistent decrease in number of fecal pellets deposited per unit of time with increasing dose in both males and females when replicates were combined. The hypothesis that the decrease in means with increasing dose may be real will be tested more adequately by the experiments of the third year in which larger numbers will be involved.

Tables 5 through 10 present the data acquired for all three strains with respect to the six quantitative traits examined. Variation in mean body weight for all three strains examined is shown in Table 5. Means, standard errors of the mean and sample sizes are presented for each replicate, sex and dose group. For strain BALB/cByJ, differences between sexes proved to be significantly different ($p \leq .01$) by factorial ANOVA. Inasmuch as differences between replicates were not significant for either sex, replicates were combined for analysis. Of interest is the fact that the mean values for even the lowest dose group, sexes compared separately, are highly significantly increased over control values on the basis of a 2-tailed t-test. In spite of the fact that body weight is known to be affected by a number of environmental variables, it is clear that under the controlled laboratory conditions employed with both the BALB/cByJ and the DBA/2J strains, this trait exhibits sufficiently low variation and the response to mutagenic effect is sufficiently high so that it provides a superb test for the effect of a suspected mutagenic agent. We would recommend that the BALB/cByJ strain be used in such tests because of the very high productivity of the animal.

With respect to strains C_3H, results are not clear, very likely due to the small numbers involved. In replicate A, there appears to be an indication of the same response observed in BALB/cByJ and, in earlier experiments, with DBA/2J. However the results in replicate B follow a different pattern in which the low mutagen dose appears to induce a decrease in body weight followed by an increase in body weight at the higher dose level. Parenthetically, the increases in body weight for replicate A are significant for both dose groups, sexes considered separately, on the basis of a 2-tailed

Table 5. Effect of TEM dose on body weight of F_1 progeny of treated male parents in inbred strains BALB/cByJ, C_3H/HeJ and DBA/2J. Males were treated at the spermatozoan germ cell stage. Standard errors are provided and sample size is given in parentheses.

A. BALB	TEM dose		
	0 mg/hg	0.1 mg/hg	0.2 mg/hg
Rep A ♂	18.23 ± .23 (62)	19.52 ± .26 (33)	20.77 ± .57 (12)
Rep B ♂	18.40 ± .23 (42)	19.75 ± .30 (29)	20.02 ± .30 (15)
Combined ♂	18.30 ± .16 (104)	19.63 ± .20 (62)**	20.35 ± .30 (27)**
Rep A ♀	16.36 ± .20 (63)	17.17 ± .24 (40)	18.25 ± .47 (18)
Rep B ♀	16.66 ± .19 (59)	17.30 ± .19 (32)	17.64 ± .24 (27)
Combined ♀	16.51 ± .14 (122)	17.23 ± .16 (72)**	17.89 ± .24 (45)**
B. C_3H			
Rep A ♂	16.97 ± .35 (22)	19.46 ± .32 (11)**	19.89 ± .20 (11)**
Rep B ♂	19.38 ± .49 (13)	18.52 ± .34 (15)	19.22 ± .37 (13)
Rep A ♀	14.87 ± .33 (11)	15.86 ± .33 (15)*	16.18 ± .22 (19)*
Rep B ♀	16.07 ± .25 (13)	15.01 ± .52 (18)	15.83 ± .91 (16)
C. DBA			
Rep A ♂	15.53 ± .41 (22)	13.71 ± 1.03 (7)	(2)
Rep B ♂	15.79 ± .42 (24)	(0)	(0)
Rep A ♀	15.00 ± .35 (12)	13.79 ± .65 (11) *	(0)
Rep B ♀	14.78 ± .38 (10)	(1)	(0)

* In comparison with control, $p < .05$
** In comparison with control, $p < .01$

t-test. In replicate B, observed differences are not significant, and it is entirely likely that an experiment involving larger numbers would have shown the same results found for the other two strains.

With respect to strain DBA/2J, only replicate A had F_1 young in a dose group other than control, but even here the numbers were too low to be meaningful. For males of replicate A, the differences between the low dose group and control are not significant based upon a 2-tailed t-test; for females of replicate A the differences between means are significant ($p < 5\%$). Obviously, where such small numbers are involved, the likelihood of a Type II error is not negligible. Since this result is in conflict with results of earlier experiments where much larger numbers were involved, we suspect that this is precisely what has happened.

In regard to the time of development of the righting response (see Table 6) results obtained with strain BALB/cByJ are persuasive and are particularly interesting in that they parallel the results obtained with strain DBA/2J in experiments of the second year. Differences between sexes only approached significance based upon a factorial ANOVA ($.05 < p < .10$). However, the differences due to treatment proved to be highly significant ($p \ll .001$). The pattern that emerges indicates an increase in the length of time between birth and development of the righting response (a decrease in rate of development) with increasing mutagen dose. Although differences between the low dose means and those of controls are not as clearly different as was the case with body weight, for each sex differences would be significant ($p \ll .05$) on the basis of a 1-tailed t-test. Based upon a 2-tailed t-test, the difference between the control and low dose means approaches significance for males replicates combined. The same difference is highly significant for females. For both sexes the difference between the high dose group and control, replicates combined, is highly significant ($p < .01$). This trait is unquestionably of great potential usefulness in detecting mutagenic activity, particularly when used in conjunction with body weight at weaning. While results with large numbers using strain DBA/2J in the experiments of the second year showed very similar effects, strain BALB/cByJ would clearly be the strain of choice in mutagenicity tests because of its greater productivity.

Data obtained for strain DBA/2J in the present experiment are based upon such limited numbers that the results are not of great usefulness. There was a face value increase in developmental time with increasing dose for both sexes of replicate A, but sample sizes are such as to make meaningful conclusions impossible. With respect to strain C_3H, a very interesting phenomenon was observed which may be meaningful in spite of the small numbers involved, if not useful. For both sexes in both replicates there was observed an increase in rate of development, a decrease in the length of time between birth and the righting response, at the lowest TEM dose. Differences between replicates were not significant. Accordingly replicates were combined. The difference between the means of the low dose group and those of control proved to be nonsignificant for males ($p \sim .15$) but highly significant for females ($p < .01$) on the basis of a 2-tailed t-test. At the highest dose developmental time remained lower than that of controls but

Table 6. Effect of TEM dose on time of development of the righting response of the F_1 progeny of treated male parents in inbred strains BALB/cByJ, C_3H/HeJ and DBA/2J. Males were treated at the spermatozoan germ cell stage. Standard errors are provided and sample size is given in parentheses.

A. BALB

	0 mg/hg	0.1 mg/hg	0.2 mg/hg
Rep A ♂	9.52 ± .14 (62)	9.73 ± .31 (33)	10.75 ± .68 (12)
Rep B ♂	9.16 ± .24 (24)	9.89 ± .28 (29)	10.53 ± .52 (15)
Combined ♂	9.38 ± .13 (104)	9.81 ± .21 (62)	10.63 ± .42 (27) **
Rep A ♀	9.76 ± .21 (63)	10.60 ± .24 (40)	11.44 ± .41 (18)
Rep B ♀	9.29 ± .23 (59)	10.25 ± .24 (32)	10.48 ± .49 (27)
Combined ♀	9.53 ± .15 (122)	10.44 ± .17 (72) **	10.87 ± .29 (45) **

B. C_3H

Rep A ♂	7.73 ± .29 (22)	7.09 ± .37 (11)	7.64 ± .51 (11)
Rep B ♂	7.85 ± .32 (13)	7.40 ± .36 (15)	7.00 ± .45 (13)
Combined ♂	7.77 ± .21 (35)	7.27 ± .26 (26)	7.29 ± .34 (24)
Rep A ♀	8.64 ± .31 (11)	6.93 ± .33 (15)	7.65 ± .37 (20)
Rep B ♀	7.77 ± .50 (13)	7.00 ± .32 (18)	7.00 ± .43 (16)
Combined ♀	8.17 ± .31 (24)	6.97 ± .23 (33) **	7.36 ± .28 (36)

C. DBA

Rep A ♂	7.77 ± .31 (22)	8.00 ± .63 (8)	9.50 ± .5 (2)
Rep B ♂	8.50 ± .31 (24)	11.00 ± 0 (2)	(0)
Rep A ♀	8.25 ± .39 (12)	8.82 ± .42 (11)	(0)
Rep B ♀	8.30 ± .30 (10)	(1)	(0)

** In comparison with control, $p < .01$

appeared to increase slightly above the low dose group. The interesting suggestion that emerges from these data is that the fitness of strain C_3H F_1 young may have been increased slightly, as judged by an increased rate of development, by the genetic variance induced at the low mutagen dose. While this hypothetical effect might or might not be of usefulness in the detection of mutagenic effects, the implications for the broader significance of mutagen induced genetic variance are very important. A similar effect was observed in the experiments of the prior year with respect to the low dose group employing strain DBA/2J. In our report of those results we noted that "...the increase in rate of development, i.e. reduction in time of development of the righting response, associated with the low mutagen dose group approaches significance in comparisons with the control. We have observed this pattern before in respect to other traits, and it may well be real." In the same experiments, among the F_2 generation young of the spermatozoan treated group the increase in rate of development (decrease in time of development) of the righting response for the low TEM dose group was even more strikingly demonstrated. With sexes and replicates combined a multiple range test indicated that the difference between the low dose group and control means was significant.

We reported then and we still feel that the simplest interpretation of these results is that the slightest amount of mutagen induced variation has the effect of shortening developmental time appreciably in some highly inbred mice strains. With increasing mutagen effect, i.e., genetic damage, developmental times are not so strikingly reduced or may be increased. This interpretation is based upon the assumption that a very limited amount of mutagen induced polygenic variance may provide a genetic buffering effect that results in more rapid development. With increasing mutagenic damage, however, the cumulative effects of the deleterious mutations will gradually mask the buffering effects of polygenic mutations. Rate of development is a phenomenon closely related to fitness, and it might be predicted that other fitness traits would show a similar pattern where the direction of "bad" or "good" effect can be guessed in a relatively straightforward manner.

With respect to the defecation portion of the open field test, the results that emerged, while not significant, are interesting and will be tested in future experiments (Table 7). With strain BALB/cByJ differences between replicates were not significant. Accordingly, replicates were combined and a factorial ANOVA (sex by dose) was carried out to determine if an apparent increase in defecation rate with increasing mutagen dose was real. While the differences between sexes proved to be significant ($p < .025$), differences due to mutagen dose were not ($p \sim .15$). Experiments currently in progress and to be conducted in the coming year may well demonstrate a mutagenic effect with respect to this trait. Nevertheless, it seems likely that the numbers required to demonstrate significance will make the trait somewhat less useful than body weight and righting response.

There were indications in strain C_3H of a reduction in defecation rate with increasing mutagen dose but the differences are so modest and the numbers so low that meaningful comparisons are not possible (Table 7). Experiments with defecation rate in strain DBA/2J failed to show differences among dose groups, although a highly significant difference between

Table 7. Effect of TEM on the defecation portion of the open field test of F_1 progeny of treated male parents in inbred strains BALB/cByJ, C_3H/HeJ and DBA/2J. Males were treated at the spermatozoan germ cell stage. Standard errors are provided and sample size is given in parentheses.

A. BALB	TEM dose		
	0 mg/kg	0.1 mg/kg	0.2 mg/kg
Rep A ♂	5.00 ± .27 (67)	4.09 ± .42 (33)	3.75 ± .35 (12)
Rep B ♂	4.36 ± .30 (42)	5.07 ± .37 (29)	4.60 ± .49 (15)
Combined ♂	4.75 ± .21 (109)	4.55 ± .29 (62)	4.22 ± .32 (27)
Rep A ♀	4.14 ± .28 (63)	4.15 ± .34 (40)	3.61 ± .49 (18)
Rep B ♀	4.39 ± .23 (59)	3.59 ± .33 (32)	3.81 ± .26 (27)
Combined ♀	4.26 ± .18 (122)	3.90 ± .24 (72)	3.73 ± .25 (45)
B. C_3H			
Rep A ♂	3.36 ± .37 (22)	3.27 ± .59 (11)	3.09 ± .49 (11)
Rep B ♂	3.31 ± .26 (13)	3.20 ± .47 (15)	3.36 ± .29 (14)
Combined ♂	3.34 ± .25 (35)	3.23 ± .36 (26)	3.24 ± .27 (25)
Rep A ♀	3.36 ± .58 (11)	2.73 ± .49 (15)	2.90 ± .35 (20)
Rep B ♀	2.69 ± .31 (13)	2.83 ± .23 (18)	3.07 ± .43 (14)
Combined ♀	3.00 ± .31 (24)	2.79 ± .25 (33)	2.97 ± .27 (34)
C. DBA			
Rep A ♂	2.32 ± .49 (22)	4.00 ± 1.05 (7)	(2)
Rep B ♂	2.79 ± .38 (24)	(2)	(0)
Rep A ♀	3.58 ± .51 (12)	2.00 ± .65(11)	(0)
Rep B ♀	1.80 ± .39 (10)	(1)	(0)

sexes was found in experiments of the past year. As can be seen in Table 7, the numbers available for strain DBA/2J in experiments of the present year are not adequate to provide meaningful conclusions.

With respect to hematocrit, results of experiments of the present year are shown in Table 8. For strain BALB/cByJ the pattern of means that emerges is erratic. While two of the differences between means are significant on the basis of a 2-tailed t-test, it would be difficult to interpret both of the significant differences as meaningful biologically. In experiments of the past two years employing strain DBA/2J, it was observed that the hematocrit reading was always higher in the middle and high mutagen doses than in the control. In only one comparison was a significant difference found, but the pattern was consistent. In the experiments of the present year involving BALB/cByJ, the high mutagen dose is associated with a higher hematocrit level for males and females of replicate A, but only males in replicate B show the same trend. By contrast, females show a significant reduction in hematocrit level ($p < .05$) in the highest dose group. The hematocrit pattern determined for strain C_3H is quite interesting in that it is reminiscent of the results with righting response. With replicates combined, both males and females show a reduction in the lowest TEM dose group below controls followed by an increase in the high dose group to a level above controls. Based upon a 2-tailed t-test analysis, replicates combined, the decrease in hematocrit from control to low dose mean is not significant for either sex (although the same difference is found in both sexes of each replicate), but the increase from low dose group to high dose group is highly significant. Again, while this pattern may not be particularly useful for the purpose of ascertaining mutagenic activity, it is of great interest as it suggests something about the phenotypic effect of small amounts of mutagen induced genetic variance.

Again, the data from the strain DBA/2J are too limited to say more than that the means are not in conflict with results of experiments of the prior years.

Data acquired with respect to tail length are represented in Table 9. Unlike earlier results with strain DBA/2J in which variation in tail length appeared to be strongly positively correlated with variation in body weight, no effect could be observed in strain BALB/cByJ in tail length variation in spite of the clear evidence of mutagenic effect on body weight. The results obtained in strain C_3H are quite interesting although the small sample sizes prevent the unequivocal demonstration of effects that may be present (Table 9). However, in each sex of both replicates, tail length is reduced in the low TEM dose group below control and increased above the low TEM dose group in the high TEM dose group. A factorial analysis of variance, sex by dose, replicates combined, indicates that the reduction in tail length in the low TEM dose group is highly significant ($F_{1, 115} = 10.13$; $p < .005$). The high dose groups are not significantly different from controls, but the increase over the low dose group is significant ($F_{1, 117} = 4.12$; $p < .05$). In view of the modest numbers involved in these comparisons, it is entirely possible that strain C_3H, employing tail length as a trait, could prove to be particularly sensitive in the detection of very low level mutagenic activity. Unfortunately, present plans do not

Table 8. Effect of TEM dose on hematocrit in F_1 progeny of treated male parents in inbred strains BALB/cByJ, C_3H/HeJ and DBA/2J. Males were treated at the spermatozoan germ cell stage. Standard errors are provided and sample size is given in parentheses.

A. BALB	TEM dose		
	0 mg/kg	0.1 mg/kg	0.2 mg/kg
Rep A ♂	49.06 ± .15 (62)	49.48 ± .28 (33)	50.50 ± .58 (12)*
Rep B ♂	49.64 ± .25 (42)	48.93 ± .21 (28)	49.87 ± .46 (15)
Rep A ♀	48.54 ± .15 (63)	48.45 ± .23 (40)	48.89 ± .31 (18)
Rep B ♀	49.50 ± .21 (32)	48.44 ± .17 (32)	48.07 ± .18 (27)*
B. C_3H			
Rep A ♂	45.91 ± .29 (22)	45.82 ± .38 (11)	46.64 ± .39 (11)
Rep B ♂	46.54 ± .45 (13)	45.87 ± .35 (15)	47.23 ± .50 (13)
Combined ♂	46.14 ± .25 (35)	45.85 ± .25 (26)	46.96 ± .32 (24)**
Rep A ♀	46.30 ± .47 (10)	46.00 ± .45 (15)	46.70 ± .25 (20)
Rep B ♀	46.08 ± .29 (13)	45.39 ± .29 (18)	46.40 ± .31 (15)
Combined ♀	46.17 ± .26 (23)	45.67 ± .26 (33)	46.57 ± .19 (35)**
C. DBA			
Rep A ♂	47.77 ± .20 (22)	48.00 ± .37 (6)	48.00 ± 1.0 (2)
Rep B ♂	48.17 ± .33 (24)	50.50 ± .50 (2)	(0)
Rep A ♀	47.17 ± .30 (12)	46.73 ± .45 (11)	(0)
Rep B ♀	46.90 ± .43 (10)	(1)	(0)

* In comparisons with control, $p < .05$

** In comparisons with low dose group, $p < .01$

Table 9. Effect of TEM dose on tail length of F_1 progeny of treated male parents in inbred strains BALB/cByJ, C_3H/HeJ and DBA/2J. Males were treated at the spermatozoan germ cell stage. Standard errors are provided and sample size is given in parentheses.

	TEM dose		
	0 mg/kg	0.1 mg/kg	0.2 mg/kg
A. BALB			
Rep A ♂	83.44 ± .31 (61)	83.22 ± .66 (27)	82.67 ± 1.40 (12)
Rep B ♂	82.15 ± .40 (41)	81.52 ± .27 (29)	83.00 ± .47 (15)
Rep A ♀	81.31 ± .33 (35)	81.90 ± .35 (40)	82.41 ± .45 (17)
Rep B ♀	81.26 ± .23 (58)	81.47 ± .26 (32)	81.15 ± .40 (26)
B. C_3H			
Rep A ♂	80.59 ± .60 (22)	78.55 ± .61 (11)	78.91 ± .79 (11)
Rep B ♂	78.42 ± .69 (12)	77.60 ± .58 (15)	78.38 ± .63 (13)
Combined ♂	79.82 ± .49 (34)	78.00 ± .43 (26)**	78.63 ± .49 (24)*
Rep A ♀	79.80 ± .49 (10)	78.47 ± .80 (15)	79.40 ± .50 (20)
Rep B ♀	77.92 ± .67 (12)	76.61 ± .66 (18)	77.64 ± .43 (14)
Combined ♀	78.77 ± .46 (22)	77.45 ± .53 (33)**	78.68 ± .37 (34)*
C. DBA			
Rep A ♂	73.82 ± .55 (17)	74.40 ± .68 (15)	
Rep B ♂	76.00 ± .48 (18)	(1)	
Rep A ♀	75.12 ± .58 (8)	73.57 ± .84 (7)	
Rep B ♀	74.33 ± .95 (6)	(1)	

* In comparisons with low dose group, $p < .05$
** In comparisons with control, $p < .01$

permit the retesting of this exciting possibility.

Data on variation in tail length in strain DBA/2J are also provided in Table 9 but, as with other traits, the small numbers available do not permit meaningful conclusions.

Finally, with respect to brain weight, a trait which looked particularly exciting in the experiments of the second year with strain DBA/2J, effects in BALB/cByJ were found for males, replicates combined, but not for females (Table 10). In males, both the low and the high TEM dose groups exhibited significantly higher brain weights than the control based upon a 2-tailed t-test. In females, however, differences among groups were not significant. It may be noted that numbers are lower in analysis of brain weight than for other traits. This is because some males were employed in translocation tests and were too old by the time the tests had been completed to have their brain weights included. Another factor, some error variance with respect to brain weight is added because of the necessity of letting females wean their litters. Thus infertile females may have their brain weights taken at 14 weeks of age, while females carrying young to weaning may be 17 weeks of age or older. The variance introduced by this problem may well account for the lack of significance among dose groups in females in contrast with the highly significant differences among males in spite of even lower numbers. Of course, it would be quite reasonable to employ a test involving brain weights of males only in strain BALB/cByJ as an adjunct to the body weight and righting response traits already demonstrated. With respect to strain C_3H (where some males were also involved in translocation tests and where the weaning problem with females also existed) the small numbers involved make meaningful conclusions impossible. The same is true with respect to strain DBA/2J in which numbers are even lower that is usually the case.

Discussion. The conclusions of major importance that emerge from these results with respect to the F_1 young of three inbred mouse strains include the conclusion that strain BALB/cByJ not only exhibits a response to mutagenic action in certain of its quantitative traits,but that it is sufficiently highly productive to be employed economically as a test animal in detecting mutagenic activity at relatively modest levels. It is emphasized that the kinds of mutations that are involved are what have been termed polygenic mutations, that is mutations of very slight effect which may be passed on from one to the next generation and,thus,be cumulative in their effects over time. By and large, most other mutagenic assays in common usage do not detect this very important type of lesion.

The most important developments of these experiments are that the effects of mutagenic treatment on the traits body weight at weaning and time of the development of the righting response in strain BALB/cByJ can be most useful as indicators of mutagenic activity of suspect compounds. Employed in conjunction with the less strikingly affected defecation portion of the open field test and brain weight in male progeny only, it seems relatively certain that the basis for a very straightforward test of mutagenicity is at hand.

Table 10. Effect of TEM dose on brain weight of F_1 progeny of treated male parents in inbred strains BALB/cByJ, C_3H/HeJ and DBA/2J. Males were treated at the spermatozoan germ cell stage. Standard errors are provided and sample size is given in parentheses.

	TEM dose		
A. BALB	0 mg/kg	0.1 mg/kg	0.2 mg/kg
Rep A ♂	476.20 ± 1.76 (60)	481.82 ± 2.32 (22)	498.00 ± 8.25 (5)
Rep B ♂	469.83 ± 3.49 (36)	479.22 ± 1.96 (23)	472.78 ± 3.08 (9)
Combined ♂	473.81 ± 1.73 (96)	480.49 ± 1.51 (45)**	481.79 ± 4.75 (14)**
Rep A ♀	487.84 ± 2.23 (58)	494.72 ± 2.32 (37)	479.82 ± 6.28 (11)
Rep B ♀	481.59 ± 1.95 (58)	478.31 ± 5.75 (32)	484.46 ± 3.58 (26)
Combined ♀	484.72 ± 1.54 (116)	487.10 ± 3.08 (69)	483.08 ± 3.11 (37)
B. C_3H			
Rep A ♂	441.36 ± 2.36 (14)	442.43 ± 4.62 (7)	440.14 ± 6.34 (7)
Rep B ♂	436.18 ± 2.98 (11)	435.40 ± 3.86 (10)	440.29 ± 4.70 (7)
Rep A ♀	447.70 ± 2.97 (10)	446.07 ± 3.80 (15)	444.79 ± 2.65 (19)
Rep B ♀	449.08 ± 3.23 (12)	439.39 ± 6.41 (18)	439.50 ± 2.75 (14)
C. DBA			
Rep A ♂	417.81 ± 1.92 (21)	396.80 ± 13.63(5)	(2)
Rep B ♂	413.50 ± 2.36 (22)	(0)	(0)
Rep A ♀	427.13 ± 1.84 (8)	429.91 ± 2.96 (11)	(0)
Rep B ♀	426.70 ± 3.11 (10)	(1)	(0)

** In comparisons with control, $p < .01$

The interesting parallel in strain C_3H evident in several traits (time of development of the righting response, hematocrit level and tail length), involving a decrease below control in trait value at the low mutagen dose level followed by an increase in trait value at the high mutagen dose level, is very interesting from the standpoint of basic genetic significance. While usefulness as an indicator of mutagenic activity is not clearly indicated, potential usefulness in ascertaining very low mutagenic activity cannot be ignored.

As indicated above, this report is of necessity limited to F_1 generation results. It is entirely possible that the data we are now acquiring with respect to strain C_3H and BALB/cByJ for F_2 generation young and, particularly, for spermatogonial treatment effects may be most exciting based, as they will be, upon greater numbers of animals.

With respect to plans for the year now in progress, the data analyzed in this report as well as data on F_2 young in the analysis of spermatozoan treatment effect and F_1 and F_2 generation progeny in spermatogonial treatment effects will be submitted to complete computer analysis. This will include partitioning of the effects of litter size in addition to the examination of differences in means due to sex, treatment and replicate.

Early in the past year, as soon as it was evident that our productivity of F_1 young was going to be quite low, we made a decision to initiate a second experiment during the year involving the BALB/cByJ strain carried to F_1 young only. These data will become available shortly and will provide a timely test of our results reported on here for strain BALB/cByJ.

With respect to plans for the experiments of the final year of the contract just beginning, employing the same design employed in past years and including all the traits employed in the past year, it is proposed that we examine the effects of TEM on the single strain BALB/cByJ in a concerted effort to demonstrate as conclusively as possible the usefulness of quantitative traits as indicators of mutagenic effect. In the interests of having test groups as large as possible, we plan to employ mutagen doses of 0, .1 and .2 mg of TEM per kg of body weight as in the past year.

As reported in the last semi-annual report, results of our first two years experiments provided substantial evidence that a late dominant lethal (perinatal) effect originally observed by Soares was responsible for a significant number of deaths in treated groups. Dr. Jack Favor had developed supporting evidence in the course of his doctoral research. These results have been submitted for publication as a manuscript with resulting favorable recommendations for publication. The manuscript is currently being revised for resubmission.

Respectfully submitted,

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Project Director

JWCJr:dm

G-32-621

REPORT OF PROGRESS FOR RESEARCH CONTRACT NIEHS NO. NO1-ES-5-2135

June 25, 1978 through December 25, 1978

In the Report of Progress for the period ending June 25, 1978, preliminary analyses of the dominant lethal and quantitative trait data of Experiment III were included. Because of the difficulty of obtaining the numbers required of the strains being tested, the experiment was initiated late, and data collection was not completed by the end of the contract year. Experiment IV, a limited repetition of part of Experiment III, had been set up later in that same period. An outline of that experiment, including numbers of animals and a schedule of matings, was also included in the last Report of Progress. Detailed statistical analyses are now available for the quantitative data of Experiment III, and these results will be presented in the second part of the present document. The first part of this document will be devoted to the description of Experiment V initiated in the present year.

Experiment V. This experiment is devoted primarily to the effects of a mutagenic agent in determining the expression of quantitative traits in a single strain of inbred mouse, BALB/cByJ. As pointed out in the last semi-annual report, a decision had been made by the Principal Investigator and Contract Officer to devote total effort in the final contract year to the testing of this highly productive strain. As in the series of preceding experiments, triethylene melamine (TEM) was the mutagenic agent employed. Again, in order to maximize the number of animals in each group only two doses (.1 and .2 mg TEM per kilogram of body weight) were employed in addition to a control group. As has been the practice since Experiment II, the number of matings set in each dose group was adjusted to compensate for dominant lethal effects of treatment in the high dose groups in such a way that similar numbers of F_1 progeny would be produced in all groups. TEM treatment effects will be tested on spermatozoanand spermatogonial stages of spermatogenesis in progeny of the F_1 and F_2 generations.

The quantitative traits which are being studied in Experiment V to determine the effect of mutagenic treatment on the F_1 and F_2 generation progeny of treated males include: 1) time of development of the righting response, 2) body weight at weaning, 3) the defecation portion of the open field test, 4) tail length at seven weeks of age, 5) hematocrit at seven weeks, and 6) brain weight at approximately 13 1/2 weeks of age. In addition to these continuously varying traits, productivity traits are also being studied to determine if treatment has been effective. These traits include 1) percent dead implantation scars of total scars, 2) total implantation scars, 3) percent females born, 4) total young born, 5) percent females weaned and 6) total young weaned. Traits 1 and 2 which are based upon inspection of uterine scars in parental generation females were collected for F_1 generation young only. Other productivity traits were obtained in both F_1 and F_2 generations.

Design of Experiment. In Experiment V, female parents used to assess the effects of treatment at different germ cell stages were assigned to one of six replicates. Replicates A, B and C, carried out two weeks apart, consisted of females mated to males which had been exposed to mutagenic treatment immediately prior to mating, thus involving mutagenized spermatozoa. These matings involved trios of females mated with each male. Replicates D, E and F were temporally separated replicate groups of females which were mated to males which had been exposed to mutagenic treatment over eight weeks prior to establishment of mating. Accordingly, the germ cells involved were mutagenized as spermatogonial cells. In these latter replicates, the spacing was irregular for technical reasons with about two and one-half weeks elapsing between Replicates D and E and about six weeks separating E and F. Replicates A, B and C involved a total of 214 male mice of the BALB/cByJ strain. In order to maximize numbers of matings, males were selected from three different age groups involved and varied from 13 to 19 weeks at treatment. Care was taken to assure that all age groups were approximately equally represented in the control and treatment groups. The three replicates were also approximately equal in numbers of matings involved.

In Replicate D crosses, thirty males were each mated with two females for a period of two weeks. In like manner, thirty males were mated each with two Replicate E females for a period of two weeks. Replicate F will be established with similar numbers of males about January 10, 1979. In total, about 175 females will be involved in testing the effects of mutagenic treatment on spermatogonial stage germ cells.

As has been general practice, females of matings involved in spermatozoa treatment tests were isolated from each other 10 days after the initiation of matings and checked daily for the presence of a litter through day 21 after the termination of matings. Young were sexed and marked by toe clipping for identification at birth. Starting on day 5, progeny were checked for the ability to right (righting response) and were checked daily until the trait appeared. All animals were weaned at four weeks of age, at which time body weight and sex were recorded, and individuals were permanently marked by coded ear punching. The defecation portion of the field test was conducted on mice at five weeks of age, while tail length and hematocrit measures were taken for mice at approximately seven weeks of age.

At the present time, F_1 young of Replicates A and B have been weaned and the fecal test has been conducted for them. The young of Replicate C are due to be weaned early in January. The young of Replicate D have been weaned and the fecal tests conducted for them, and the young of Replicate E are scheduled to be weaned early in January. As was pointed out above, Replicate F matings have not yet been established.

It is planned that matings of F_1 individuals to produce F_2 progeny will be established for all replicates between eleven and twelve weeks of age. Pair crosses will be established between individuals taken randomly, except that sibling crosses will be precluded, within dose and replicate. Three replicates of spermatozoan treatment groups and three of spermatogonial treatment groups will be established by the progeny of the same parental replicate groups. Table 1 summarizes the scheduling of parental and F_1 generation matings.

Table 1. Schedule of Matings for Parental Generation

	Replicate					
	VA	VB	VC	VD	VE	VF
Females received	8/9/78	8/30/78	9/20/78	9/13/78	10/4/78	10/25/78
Matings	10/19/78	11/1/78	11/13/78	10/26/78	11/14/78	to be est. ~1/10/79
Matings separated	10/26/78	11/8/78	11/20/78	11/9/78	11/28/78	to be sep. ~1/24/79

The mutagenic treatment consisted of intraperitoneal injection of triethylene melamine dissolved in .50 cc Hank's balanced salt solution prepared immediately prior to injection. Control males received the same quantity of carrier only. Doses were based on the mean body weight of a random sample of the mice to be treated. Where males of different ages were employed, mean body weights were established for each of the different age groups involved. As indicated above, different numbers of matings were established for each dose group to compensate for dominant lethal effects in the higher doses of spermatozoan treatment groups and in order to produce similar numbers of F_1 progeny. The total number of males treated in Replicates A, B and C combined, in the dose groups 0.0, .1 and .2 mg/kg of body weight were respectively 37, 62 and 115. All animals were identified by a coded number so that observations were blind with respect to dose. Because dominant lethal effects in spermatogonial treatment groups are virtually absent, Replicates D, E and F dose groups were established with approximately 30 males employed in each dose group for all three replicates combined.

II

Results: (A) Dominant Lethality, Experiment III. Preliminary discussions of dominant lethal variation among the three strains compared in Experiment III (viz. DBA/2J, BALB/cByJ and C3H/HeJ)were presented in the most recent semi-annual report. We now have statistical analyses to support the tentative conclusions of that report.

It was reported in the previous Report of Progress that "while total live scars tend to decrease with increasing dose for both DBA/2J and BALB/cByJ, strain C3H/HeJ showed an increase in live scars between controls and the lowest mutagenic dose followed by a drop to a level below that of controls for the highest mutagenic dose". Analyses of total scars was carried out by ANOVA after square root transformation. As expected, differences between means due to dose were highly significant ($p < .001$), but the strain x dose interaction that should have geen produced by the unusual performance of strain C3H/HeJ at high dose was not significant ($p \sim .195$). Differences among strains were highly significant ($p < .001$). Differences between replicates were negligible.

With respect to the traditional measurement of dominant lethality, percent dead scars of total scars, it was reported that "the BALB/cByJ strain exhibits a clear increase from a very low control value to a very high value with the highest mutagen dose supported by closely parallel and regular replicate responses. Strain C3H/HeJ is remarkable in that clear evidence of dominant lethality by the traditional measure is lacking. In both replicates the highest mutagen dose shows a face value decrease in percent dead scars of total, but all differences between experimental groups and control are slight and show no evidence of a dominant lethal effect." Percent dead scars of total were analyzed by ANOVA after arc sine transformation of data with the following results. The differences among strains were indeed highly significant ($p < .001$) due evidently to the performances of BALB/cByJ and DBA/2J.

Data from Experiments I and II are a part of a paper by Favor, Soares, and Crenshaw demonstrating a late dominant lethal effect in mice. This paper has been accepted for publication by the journal Mutation Research, with publication expected in the near future. As reported in the last Report of Progress, the results of Experiment III support our conclusions based upon earlier experiments, but the detailed statistical analyses have not yet been completed. Tables 2, 3 and 4 of the last Report of Progress submitted gave details of important dominant lethal parameters for all three strains. While some differences have emerged in the more detailed statistical analyses, the original tables are essentially correct.

Quantitative Traits, Experiment III. The numbers of animals involved in the present experiment and the amount and complexity of data acquired in Experiment III and subsequently have prevented the complete statistical analyses of all data as of the present moment. The data that we have analyzed make significant contributions toward our understanding of a number of factors involved in the use of quantitative traits in the assessment of mutagenic damage. For the first time our sample sizes have been sufficiently great so as to provide us with some definite conclusions and, perhaps equally important, they provide patterns of response that, while not necessarily significant, suggest meaningful genetic mechanisms that could account for the effect. Numbers of DBA/2J strain mice are too limited to make much sense in the present analyses. Accordingly, we will limit discussions below to strains BALB/cByJ and C3H/HeJ for the F_1 generation groups representing mutagenized spermatozoa and, for BALB/cByJ only, groups representing germ cells treated as spermatogonia.

Time of Development of the Righting Response. It was reported in the most recent Report of Progress that preliminary analyses of data revealed interesting responses in the time of development of the righting response to doses of the mutagen TEM in both strains BALB/cByJ and C3H/HeJ, but that the responses were remarkably different in the two strains. In BALB/cByJ, in both replicates and both sexes, there was a more or less linear increase in the time of development of the righting response with increasing mutagen dose. One way analyses of variance comparisons showed highly significant differences between the control and the highest dose group in males and between controls and both mutagen doses in females. Subsequently, employing covariance techniques, these data were adjusted for variation in litter size. While the pattern of an increase in time of development of the righting response with increasing dose remains the same, differences among dose groups were no longer significant.

Thus, much of the variance between dose groups in the raw data is attributable to litter size effects. An examination of the righting response in spermatogonial treatment groups reveals precisely the same pattern, an increase in time of development of the righting response with increasing dose. The differences between control and the experimental groups are not as great, but the sample size is nearly three times larger than that in the spermatozoan treatment group tested. Again, the differences among dose groups are not significant, but the suggestion is clear that mutagen induced variance in strain BALB/cByJ appears to slow down the rate of development at least as measured by time of development of the righting response. As might have been expected, the effect is not great in spermatogonial treatment groups, where some of the variance might have been eliminated, as it is in spermatozoan treatment groups. Inasmuch as the numbers involved are moderately large, it may be concluded that righting response would not be a useful trait to employ if BALB/cByJ were the strain being used. Fortunately, there are in progress two experiments that may provide additional evidence on the question.

The effects of triethylene melamine on strain C3H/HeJ were remarkably different from that of BALB/cByJ. As we reported in the last Report of Progress, strain C3H revealed a reduction in time of development of the righting response in mutagenized groups, but these differences were significant only for females at the low dose group. In contrast with the results of covariation for litter size found in strain BALB/cByJ, the effects of covariation were not significant in C3H/HeJ but, adjusting for litter size, differences among dose groups were highly significant ($p < .001$). From Table 2 it can be seen that the effect of mutagenic action was to decrease in similar manner the time of development of the righting response in both dose groups, with the control being significantly different from both. Apparently the effect of mutagen induced variation at the dose levels involved is to increase the rate of development in strain C3H/HeJ. As was pointed out in the most recent Report of Progress, "a similar effect was observed in the experiments of the prior year with respect to the low dose group employing strain DBA/2J." We also noted in an earlier Report of Progress that ".....the increase in the rate of development, i.e. reduction in time of group approaches significance in comparisons with control. We have observed this pattern before in respect to other traits, and it may well be real." We continue to feel that the simplest interpretations of these results is that

Table 2. Effect of TEM dose on time of development in days of the righting response of the F_1 progeny of treated male parents in inbred strains BALB/cByJ and C3H/HeJ. Males were treated at the spermatozoan or spermatogonial cell stages. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance. Sexes and replicates are combined for simplicity.

Strain	Stage	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
BALB	Spermatogonia	n.s.	8.95 (376)	8.97 (458)	9.11 (419)
BALB	Spermatozoa	n.s.	9.84 (226)	9.88 (134)	10.07 (72)
C3H	Spermatozoa	$p < .001$	8.25 (59)	7.04 (59)	7.09 (60)

the slightest amount of mutagen induced variation has the effect of shortening developmental time appreciably in some highly inbred mice strains. With increasing mutagen effect, i.e. genetic damage, developmental traits are not so strikingly reduced or may be increased. This interpretation is based upon the assumption that a very limited amount of mutagen induced polygenic variance in a highly inbred strain may provide a genetic buffering effect that results in more rapid development. With increasing mutagen damage, however, the cumulative effects of the deleterious mutations will gradually mask the buffering effects of polygenic mutations. Rate of development is a phenomena closely related to fitness, and it might be predicted that other fitness traits would show a similar pattern where the direction of "bad" or "good" effect can be guessed in a relatively straightforward manner." By this interpretation it is suggested that BALB/cByJ is somewhat better balanced genetically than C3H/HeJ and that the general effect of added variance on the former is deleterious and reduces rate of development. By contrast, strain C3H is interpreted as being less well balanced genetically, perhaps more highly inbred or, at least, less well balanced, and is "improved" by added genetic variation as indicated by an increase in rate of development. It will be most interesting to study the effects on spermatogonial mutagenized stages in C3H which will be forthcoming shortly. It can be predicted that a similar effect would occur. That is, an increase in rate of development in mutagenized groups relative to controls. Whether the effect would be greater or lesser than in spermatozoan treatment groups cannot be predicted from the data available. From a purely practical viewpoint if the results with C3H/HeJ can be confirmed, considering the modest numbers involved, this strain employing the time of development of the righting response trait, would be extraordinarily sensitive as an indicator of mutagenic activity.

Body Weight at Weaning. In the last Report of Progress it was reported that there were highly significant increases in body weight in mutagenized groups in strain BALB/cByJ and in Replicate A (Replicate B was not available) of strain C3H/HeJ. These conclusions were drawn prior to adjustment for litter size effect. Table 3 shows the results after adjustment for litter size. It can be seen that, after adjustment for litter size, differences among groups were no longer significant. Indeed, the direction of difference in means was actually reversed in strain BALB/cByJ. In strain C3H/HeJ the direction of differences remains the same as prior to adjustment for litter size. Means, adjusted for litter size, are shown for the spermatogonial stages in strain BALB/cByJ. The differences among means proved to be highly significant ($p < .001$) based upon a factorial ANOVA with both replicates of both sexes showing the same tendency for mutagenized groups to have greater body weights than controls. Combining replicates and comparing sexes separately, only the high mutagen dose group in females was significantly greater than control ($p < .001$), but in males both low and high dose groups were highly significantly greater than controls ($p < .001$ in both cases). The latter comparisons are based upon t-test analyses.

Table 3. Effect of TEM dose on body weight at weaning of F_1 progeny of treated male parents in inbred strains BALB/cByJ and C3H/HeJ. Males were treated at the spermatozoan or spermatogonial germ stages. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance. Sexes and replicates are combined for simplicity.

Strain	Stage	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
BALB	Spermatogonia	$p < .001$	16.49 (376)	16.87 (458)	17.06 (419)
BALB	Spermatozoa	n.s.	18.00 (226)	17.83 (134)	17.67 (72)
C3H	Spermatozoa	n.s.	16.93 (59)	16.97 (59)	17.36 (60)

The lack of significant differences among the groups in spermatozoan treated groups may be attributed most simply to small sample sizes. The fact that very large sample sizes are associated with the significant differences in spermatogonial treatment groups lends greater confidence to the results. Fortunately, an additional experiment already in progress will provide a test of these conclusions. As has been pointed out previously, the fact that this approach may detect differences in the spermatogonial stages is particularly exciting in view of the difficulty of measurements of mutagenic effect generally for this stage.

Tail Length at Seven Weeks of Age. Remarkably, highly significant differences were found among dose groups in spermatogonial stage treated groups in strain BALB/cByJ, but not among treatment groups in spermatozoan treated stages. (Table 4). The sample sizes were considerably greater in the spermatogonial groups, as were the differences between the several means.

Table 4. Effect of TEM dose on tail length at seven weeks of age of F_1 progeny of treated male parents in inbred strains BALB/cByJ and C3H/HeJ. Males were treated at the spermatozoan or spermatogonial germ cell stages. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined, except as noted.

Strain	Stage	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
BALB	Spermatogonia	$p < .001$	81.88 (366)	82.57 (441)	82.51 (406)
BALB	Spermatozoa	n.s.	82.04 (222)	82.26 (128)	82.07 (70)
C3H	Spermatozoa	$p < .05$	79.01 (56)	77.91 (59)	78.90 (58)
C3H	♀ Rep A		80.68 ± .58 (22)	78.55 ± .61 (11)	78.91 ± .79 (11)
	♀ Rep B		78.42 ± .69 (12)	77.60 ± .58 (15)	78.29 ± .59 (14)
	♂ Rep A		79.80 ± .49 (10)	78.47 ± .80 (15)	79.40 ± .51 (20)
	♂ Rep B		78.17 ± .57 (12)	76.61 ± .66 (18)	77.69 ± .46 (13)

The effect of mutagen treatment was to increase tail length for both dose groups, but not in a linear manner. The simplest interpretation of the difference in response in the two different germ cell stage groups is that the mutagen induced genetic variance is filtered through spermatogenesis in spermatogonial test groups. Therefore, presumably mostly lesions of minor effect are left, with the effect of increasing tail length. The more extensive and more numerous (and likely more damaging) induced variance in spermatozoan stages has produced appreciable effects in the direction of shortening and perhaps also lengthening the tail. These conclusions are based upon means adjusted for variation in litter size. It is of interest that variance due to litter size was not significant with respect to spermatozoan stage cell treatment but was significant ($p \sim .025$) in regard to spermatogonial stage group tests.

With respect to strain C3H/HeJ, significant differences were found ($p \sim .03$) among groups after adjustment for litter size in spite of the relatively small sample sizes involved (See Table 4). The effect of the mutagen induced variance was to reduce tail length in the low dose group followed by an increase to a level very slightly below that of controls in the high dose group. Raw data are also provided in Table 4 for both replicates and both sexes to demonstrate the impressive parallel in all four groups in this general pattern. Not surprisingly, a factorial analysis of variance of the unadjusted data provided a similar conclusion to that based upon data adjusted for litter size with tail lengths in the low dose group highly significantly below those of controls, and the high dose group significantly above the low dose group, but not significantly different from controls. This is a pattern that we have seen before in strain DBA/2J. It would be highly desirable to repeat this experiment with C3H/HeJ. It is evident that, if real, the high sensitivity of this strain in spermatozoan stage tests would make it extraordinarily useful and economical as a test animal employing the system presently being developed to determine mutagenic effect through quantitative trait changes. While there were highly significant effects of litter size on tail length ($p < .001$), the pattern of variation remained very similar before and after such adjustment, and the significance of differences among groups remained.

Hematocrit at Seven Weeks of Age. It is convenient to focus upon the effect of mutagenic treatment upon strain C3H/HeJ first because of the similarity in pattern of response in hematocrit to that found for tail length (Table 5). After adjustment for litter size by covariance, the differences among spermatozoa treated group means were highly significant ($p < .002$). Again there was found a pattern of reduction in hematocrit in the low dose group below that of controls followed by an increase in hematocrit in the high dose group to approximately the level of the mean. While there was a significant effect of litter size ($p < .05$), adjustment for litter size did not alter significantly this pattern. The same pattern of reduction in mean at low dose followed by increase in mean at high dose was found in both sexes of both replicates. Unadjusted means are presented in Table 5 to show this remarkable and persuasive pattern. The usefulness of this strain for possibly detecting very slight mutagenic effects is emphasized. While these results do require confirmation, one cannot ignore the potential for the strain considering the small number of organisms employed and the remarkable effect on mean differences.

Table 5. Effect of TEM dose on hematocrit at seven weeks of age of F_1 progeny of treated male parents in inbred strains BALB/cByJ and C3H/HeJ. Males were treated at the spermatozoan or spermatogonial germ cell stages. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined, except as noted.

Strain	Stage	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
BALB	Spermatogonia	$p \sim .08$ (n.s.)	48.73(365)	48.86 (437)	48.63 (410)
BALB	Spermatozoa	$p \sim .11$ (n.s.)	48.88 (226)	48.85 (133)	49.28 (72)
C3H	Spermatozoa	$p < .002$	46.30 (58)	45.71 (59)	46.61 (59)
C3H	♀ Rep A		45.91 ± .29 (22)	45.82 ± .38 (11)	46.64 ± .39 (11)
	♀ Rep B		46.54 ± .45 (13)	45.87 ± .35 (15)	47.21 ± .46 (14)
	♂ Rep A		46.30 ± .47 (10)	46.00 ± .45 (15)	46.70 ± .25 (20)
	♂ Rep B		46.08 ± .29 (13)	45.39 ± .29 (18)	46.36 ± .33 (14)

The effects of mutagenic treatment on strain BALB/cByJ are interesting but less clear cut. In neither case are there significant differences among means after adjustment for litter size. In each case effects of litter size only approached significance ($.05 < p < .10$). Similarily, effects of dose on differences among means only approached significance. In the case of spermatozoan test groups $p \sim .11$. With respect to spermatogonial groups, $p \sim .08$, and both sexes of both replicates showed a slight increase in hematocrit in the low dose group. In the high dose group, males of both replicates and females of one showed a similar decrease below controls in hematocrit. Females of the other replicate showed, by contrast, an increase in hematocrit. Clearly, no simple biological explanation emerges to account for such a pattern in spite of appreciable sample sizes. The pattern for strain BALB/cByJ spermatozoan test groups is similarly complex and was analyzed in the last Report of Progress. Means adjusted for litter size have been appended in the present report (Table 5) and show virtually no change in the low dose group in comparisons with controls. However, there was an appreciable increase in the high dose group over controls (and the low dose group) in respect to hematocrit. An interesting parallel with the results observed in spermatogonial test groups is that three of four sex/replicate groups showed an increase in hematocrit in the high dose group, while females of one replicate showed an equally appreciable decrease. This is the reverse of the pattern observed for spermatogonia, and is equally difficult to explain biologically.

We conclude that with respect to hematocrit, strain C3H/HeJ shows promise while BALB/cByJ does not, especially when one considers the very small numbers involved in the former as compared with the great numbers involved in the latter.

Defecation Portion of the Open Field Test. As indicated in the last Report of Progress, differences among groups in strain C3H/HeJ were not significant. There were no confounding effects of litter size nor were there replicate/dose/sex interactions. It is simply that the means were so slightly different and the sample sizes so small that meaningful patterns are not evident. Combining sexes and replicates and adjusting for variation in litter size, the means showed a pattern of slight decrease in fecal rate in the low dose group relative to controls followed by increase in the high dose group to approximately the control level. Since this pattern has been observed before, it would be desirable to look at defecation rate in C3H spermatozoan treatment groups where larger numbers are involved. It may very well be real.

Table 6. Effect of TEM dose on defecation rate at five weeks of age in F_1 progeny of treated male parents in inbred strain BALB/cByJ. Males were treated at the spermatozoan and spermatogonial germ cell stages. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined.

Strain	Stage	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
BALB	Spermatogonia	$p < .05$	4.03 (376)	4.36 (458)	4.38 (417)
BALB	Spermatozoa	$p \sim .20$	4.53 (226)	4.18 (134)	3.92 (72)

The data with respect to BALB/cByJ are, by contrast, considerably more interesting. With respect to the spermatozoan treatment group, differences between means are sufficiently great and the pattern of response among replicates and sexes sufficiently consistent as to suggest reality (Table 6). Fortunately, two additional experiments already in progress will provide further data to confirm or reject the evident pattern. Litter size effects were appreciable but not significant, and, except for a highly significant difference between sexes, there were no other significant interactions. The pattern that emerges, grouping sexes and replicates and adjusting for litter size effects, involves a reduction in defecation rate that is roughly linear for the two dose groups as compared with controls. While there is some irregularity in this pattern when sexes and replicates are compared seperately, combining replicates but treating sexes seperately, the pattern that emerges for males and females alike is a roughly linear reduction in defecation rate from control through low to high dose group. This latter comment is based upon comparisons of unadjusted means which are affected only slightly as a result of litter size adjustment.

In the spermatogonial treatment groups with the much larger sample sizes involved significant differences among dose groups, after adjustment for litter size effects, were found ($p < .05$). Effects of litter size were appreciable, but not significant. There was a highly significant difference between sexes, but significant interactions were not found. Most interestingly, the pattern that emerges is of an increase in means from the control group to treated groups, which are themselves approximately equal. While, at first glance it might appear that mutagen induced variance in spermatozoan germ cell stages should not be the opposite in effect of mutagen induced variance in spermatogonial germ cell stages, it is not entirely unreasonable that this is the case. It seems quite likely that mutations induced in spermatozoa may be much more numerous and involve a wider variety of lesions than mutations induced in spermatogonia and surviving the process of spermatogenesis, which would very likely include lesions of considerably reduced effect individually. In an experiment presently in progress, results will be obtained to test this initial conclusion, but the numbers leave little doubt as to the likelihood that the effect is real.

Brain Weight at Approximately Thirteen Weeks of Age. As reported in the last Report of Progress numbers were too small in strain C3H comparisons to permit any conclusions as to effect of mutagenic agent on brain size. It was pointed out that two major factors interfered with conducting a fair test of the brain weight trait. One of these has to do with the necessity of retaining females until they carried their young to weaning which introduced an element of age variation from thirteen to seventeen weeks of age or even older for some females. Similarly some males were involved in translocation tests to determine the effect of translocations upon quantitative traits. Thus, numbers of males were necessarily reduced. Although there were considerably greater numbers in the spermatozoan groups for strain BALB/cByJ than for strain C3H/HeJ, there is some evidence that the effect of age variance in females complicates considerably the interpretation of data acquired. As might have been expected, there was a highly significant effect of the litter size on brain weight. Similarly, there were highly significant differences between replicates and sexes. Even more unhappily, there was a highly significant replicate x dose x sex interaction. In spite of all this, the differences among the groups due to dose approached significance ($p < .20$), but the adjusted means are so strikingly different from the unadjusted means as to suggest the need for a more critical analysis of the data. Fortunately, two other experiments are already in progress which will shed light upon this complicated analysis.

Table 7. Effect of TEM dose on brain weight at approximately thirteen weeks of age in F_1 progeny of treated male parents in inbred strain BALB/cByJ treated at the spermatogonial germ cell stage. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined.

Strain	Stage	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
BALB	Spermatogonia	$p < .01$	473.04 (350)	476.14 (420)	474.85 (389)

The spermatogonial test groups for strain BALB/cByJ were much more satisfying. There were highly significant effects of litter size which, however, produced very modest changes in the dose mean values. There were also highly significant differences between replicates and between sexes. In spite of highly significant group x sex and significant dose x sex interactions, differences among groups remain highly significant. After adjustment for litter size, and combining sexes and replicates, the mean pattern reflects an appreciable increase from control to low dose group followed by a modest decrease to the highest dose group which is still appreciably above the control level. As a matter of fact, the most consistent element of the patterns observed, i.e. in both sexes of both replicates, is a modest increase in brain size in high dose groups with respect to controls. There is some variation in the low dose groups with males tending to show considerable increases in brain weight relative to controls. In females the mean response is more nearly linear with low dose group brain weights lying somewhere between those of controls and high dose group brain weights. However, in one replicate treated group responses were very nearly equal; in the other low dose group brain weights were below those of controls. This variation in response among females undoubtly is responsible for the replicate x sex and dose x sex interaction. The differences between control and low dose groups within females would not be significant for either replicate, nor is the difference between the female low dose group response in Replicate C significantly different from that in Replicate D. The simplest interpretation, then, is that there appears to be a linear response in females, but, in males, an increase to low dose group followed by a decrease to high dose group. In spite of these complications, the brain weight trait would still appear to be quite useful employing strain BALB/cByJ in the detection of strong mutagenic effect or, employing males only, as a sensitive detector of mutagenic effect. Fortunately, there is an additional experiment in progress which will permit us to evaluate more completely the importance of female variation in the present experiment.

Discussion. Detailed statistical analyses of the data, particularly in regard to results adjusted for litter size effects, have revealed that early optimism for the usefulness of strain BALB/cByJ was not realized. Nevertheless, the large numbers of organisms produced by this highly productive strain provide the basis for some very useful effects on quantitative traits particularly, and quite interestingly, in spermatogonial treatment groups. In this regard, highly significant and approximately linear increases in body weight with increasing mutagen dose were found after adjustment for the effects of litter size. Similarily, a highly significant though non-linear increase in tail length in mutagenized groups was found after adjustment for the effects of litter size. The increases resulting from both doses were approximately the same. This same pattern was observed in defecation rate where a significant increase between the control and each dose group was found, but in which the differences between the two mutagen doses were negligible. Finally, with respect to brain weight, highly significant differences were found among dose groups with an increase from control to low dose group followed by an appreciable decrease in the highest dose group. This pattern may be accounted for on the basis of modest amounts of induced genetic variance producing an effect in one direction, with excessive amounts producing a reversal of direction.

With respect to BALB/cByJ spermatozoan test groups, while no significant results were obtained (after adjustment for litter size effects) interesting patterns were observed with respect to several characters, viz. body weight, hematocrit, and defecation rate, and the differences among means approached significance in some cases. Two additional experiments are underway with strain BALB/cByJ which will provide a test of these observed patterns. Inasmuch as the sample sizes in the spermatogonial stage groups were nearly two and one-half times as large as those of spermatozoan test groups, it is quite possible that some of the differences observed may become significant in the additional experiments being conducted. One of these experiments will also include a spermatogonial stage group. Thus these results also will be tested.

With respect to strain C3H/HeJ, statistical analyses of the spermatozoan stage only are available at the moment. These are most exciting and suggest that it would be highly desirable to continue exploration of this strain as a potential animal of choice in a mutagenic assay test using quantitative traits. With respect to two characters, time of development of the righting response and hematocrit, highly significant differences among dose groups were found in spite of sample sizes on the order of sixty animals per dose group. The pattern in each of these two traits showed a decrease in measure at the low dose level followed by an increase to approximately the control level in the high dose group. This identical pattern was also found in tail length which was accompanied by a significant difference among means. These statistical indications of significance were all found after adjustment for litter size effects. It seems

reasonable to expect that, while strain C3H/HeJ might not be too reliable as an indicator of high mutagenic activity, it may provide an extremely sensitive indicator in that it responds very strongly to low levels of mutagenic activity. Since confirmation of these results is of great importance before they can provide the basis for a useful assay system, the potential usefulness of this organism makes it very desirable to do so. Statistical analyses of the spermatogonial stage results for strain C3H/HeJ have not yet been completed, but on the assumption that limited amounts of induced genetic variance produce major effects, it can be predicted that results with the spermatogonial stage will be most interesting.

With respect to plans for the year now in progress, we will continue to acquire data on Experiment V, the final experiment to be carried out under this research contract. These data will be submitted to complete statistical analyses including partitioning of the effects of litter size in addition to the examination of differences in means due to sex, treatment, replicate and interactions of these factors.

We are also continuing the analyses of results of data acquired in Experiment III and Experiment IV, the latter a limited test involving repetition of the initial stages of Experiment III only.

Finally a collaborative effort between Dr. Eugene Soares, Dr. John Favor and the undersigned with respect to a late dominant lethal (perinatal) effect in mice has been accepted for publication by Mutation Research and should be out very shortly. This study was based upon results of Experiments I and II in this series and upon separate studies by Soares and Favor carried out earlier.

Respectfully submitted,

John W. Crenshaw, Jr.

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Project Director

G-32-621

FINAL REPORT FOR RESEARCH CONTRACT NIEHS NO. NO1-ES-5-2135

December 25, 1978 through June 25, 1979

In the last two Reports of Progress for the year ending December 25, 1978, three experiments (III, IV and V) were dealt with in part. The primary reason for this incomplete treatment was that we initiated experiments with a highly productive inbred mouse strain, BALB/cByJ. This decision produced some very exciting results but cramped our facilities to the extent that we were forced to alter original schedules for the completion of experiments. This delay also lead to our request for an extension of the contract without funds.

Experiment III has been described in detail, and statistical analyses of the dominant lethal results and the mutagenic effects on quantative traits have been described for the F_1 generation groups representing mutagenized spermatozoa and spermatogonia. In this final report we will complete the discussion of results on quantitative trait analysis for Experiment III for the F_2 generation groups representing both mutagenized spermatozoa and spermatogonia.

Experiment IV, representing a limited repetition of Experiment III, including only F_1 generation groups treated as spermatozoa, has been outlined previously. Statistical analyses of dominant lethal data and the results of quantitative trait analyses will be presented here.

Finally, Experiment V was outlined in the previous Report of Progress in respect to experimental design, numbers of parental animals and a schedule of matings up to but not including the establishment of F_1 crosses. Mating schedules for this experiment, dominant lethal data and the results of quantitative trait analyses will be added in the present report.

The three experiments to be discussed in this report are devoted primarily to the effects of a single mutagenic agent in altering the expression of quantitative traits in the inbred mouse strain, BALB/cByJ. In Experiment III, two other inbred mouse strains were also considered (DBA/2J and C3H/HeJ), but the numbers of young produced by these strains were so low as to provide results that must be confirmed to be useful. In all three experiments the mutagenic agent employed was triethylenemelamine (TEM). In order to maximize the number of animals in each group, only two doses (.1 and .2 mg TEM per kilogram of body weight) were employed in addition to a control group. In all these experiments the number of matings set in each dose group was adjusted to compensate for dominant lethal effects of treatment in the high dose groups in such a way that similar numbers of F_1 progeny would be produced in all treatment groups. Treatment effects were tested in spermatozoan and spermatogonial stages

of spermatogenesis in progeny of the F_1 and F_2 generations, except that in Experiment IV only F_1 generation spermatozoan treated young were produced.

In all three experiments, the quantitative traits studied to determine the effects of mutagenic treatment were: 1) time of development of the righting response, 2) body weight at weaning, 3) the defecation portion of the open field test, 4) tail length at seven weeks of age, 5) hematocrit at seven weeks, and 6) brain weight. The time at which brain weights were taken was variable depending upon the desirability of obtaining data which precluded the early sacrifice of an individual. However in Experiments IV and V brain weights were taken at approximately 15 weeks of age for males and approximately 19 weeks of age for females.

In addition to the continuously varing traits listed above, productivity traits were also studied to determine if treatment had been effective. These traits included: 1) percent dead implantation scars of total scars, 2) total implantation scars, 3) percent females born, 4) total young born, 5) percent females weaned and 6) total young weaned. Traits 1 and 2, which are based upon inspection of uterine scars in parental generation females, were analyzed for F_1 generation young only. Other productivity traits were studied in both F_1 and F_2 generations.

As has been general practice, females of crosses involved in spermatozoan treatment tests were isolated from each other ten days after the initiation of matings and checked daily for the presence of a litter through day 21 after the termination of matings. Young were sexed and marked by toe clipping for identification at birth. Starting on day 5, progeny were checked for the ability to right themselves and were checked daily until the trait appeared. All animals were weaned at four weeks of age, at which time body weight and sex were recorded, and individuals were permanently marked by coded ear punching. The defecation portion of the field test was conducted on mice at five weeks of age; tail length and hematocrit measures were taken for mice at approximately seven weeks of age. As indicated above, brain weights were taken at about 15 weeks of age for males and at about 19 weeks of age for females.

For all experiments, the mutagenic treatment consisted of intraperitoneal injection of triethylenemelamine carried in .25 cc Hank's balanced salt solution prepared immediately prior to injection. Control males received the same quantity of carrier only. Doses were based on the mean body weight of a random sample of the mice to be treated.

Results: Quantitative Traits, Experiment III. Results of analyses of data for both spermatozoan and spermatogonial treated groups of the F_1 generation were considered in the last Report of Progress. In this report, analyses of data with respect to the F_2 generation for spermatozoan and spermatogonial treated groups for strain BALB/cByJ will be discussed. Where meaningful comparisons can be made, the results of earlier analyses have been repeated.

Time of Development of the Righting Response. In the last Report of Progress no significant differences were reported among dose groups for this trait for either spermatozoan or spermatogonial series of the F_1 generation. However, in both cases it was noted that there was a roughly linear increase in the time of development of the righting response with increasing mutagen dose. In the F_2 generation of the spermatozoan treated group the same situation obtains, i.e., there is an increase in time of development of the righting response with increasing dose. Again the relationship is roughly linear and the differences are not significant ($p < .10$).

With respect to the spermatogonial treated series in the F_2 generation, differences among dose groups were not significant. Interestingly, the pattern is not consistent with that observed in other germ cell/generation groups. The face value differences among means involve a very slight increase in time of development of the righting response from the control to the low dose group followed by an appreciable decrease in time of development of righting response between the low dose and high dose group. As indicated earlier, "it may be concluded that righting response would not be a useful trait to employ if BALB/cByJ were the strain being used."

Body Weight at Weaning. In the most recent Report of Progress it was reported that when means were adjusted for litter size there were highly significant differences ($p < .005$) among the F_1 spermatogonial dose groups, but differences among dose group means in the F_1 generation spermatozoan treatment series were not significant. Table 1 is taken in part from this source but includes, in addition, data for the F_2 generation spermatozoan and spermatogonial treatment groups. As may be seen, significant differences were found among dose group means for both of these treatment groups, with the more impressive differences being found among the spermatogonial treatment means.

* * *

Table 1. Effect of TEM dose on body weight at weaning of F_1 and F_2 progeny of treated male parents in inbred strain BALB/cByJ in Experiment III. Males were treated at the spermatozoan or spermatogonial germ cell stages. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance. Sexes and replicates are combined for simplicity.

Strain	Stage	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
BALB	F_1 Spermatozoa	n.s.	18.00 (226)	17.83 (134)	17.67 (72)
BALB	F_2 Spermatozoa	$p < .05$	17.17 (641)	17.45 (270)	17.10 (98)
BALB	F_1 Spermatogonia	$p < .005$	16.49 (376)	16.87 (458)	17.06 (419)
BALB	F_2 Spermatogonia	$p < .01$	16.84 (462)	17.17 (466)	17.11 (509)

The results indicate that body weight may be a very useful character employing BALB/cByJ, with appreciable increases in body weight expected from relatively weak mutagenic doses in the F_2 generation only of the spermatozoan treatment group but in the F_1 and F_2 generation groups of spermatogonial treated groups. As has been pointed out previously, the fact that this approach detects differences in the spermatogonial

stages is particularly exciting in view of the difficulty of measurement of mutagenic effect generally for this stage. The biological significance of these results will be discussed at greater length later in this report.

Tail Length at Seven Weeks of Age. As stated in the last Report of Progress there were no significant differences among dose group means in the F_1 generation spermatozoan treatment group, but highly significant differences were found among dose groups in the F_1 generation spermatogonial treatment group (Table 2). The same pattern emerges in the F_2 generation groups in that the spermatozoan treatment groups show no significant differences, while the spermatogonial groups show significant differences that closely parallel those of the F_1 spermatogonial group in pattern. In each case there is an impressive increase in length of tail in the low dose groups with respect to the tail length mean in controls. This is followed by a slight, nonsignificant, reduction in tail length at the highest dose level in both the F_1 and F_2 spermatogonial groups. This parallels precisely the pattern for the same groups in body weight. The close correspondence between tail length effect and body weight effect has been observed in a number of other experiments in this series. Although it seems likely that body weight and tail length are associated and not independent, the two traits employed together would increase reliability of conclusion when they pointed in the same direction.

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Table 2. Effect of TEM dose on tail length at seven weeks of age of F_1 and F_2 progeny of treated male parents in inbred strain BALB/cByJ in Experiment III. Males were treated at the spermatozoan or spermatogonial germ cell stages. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined.

Strain	Stage	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
BALB	F_1 Spermatozoa	n.s.	82.04 (222)	82.26 (128)	82.07 (70)
BALB	F_2 Spermatozoa	n.s.	80.88 (613)	80.92 (264)	80.66 (96)
BALB	F_1 Spermatogonia	$p < .001$	81.88 (366)	82.57 (441)	82.51 (406)
BALB	F_2 Spermatogonia	$p < .05$	79.60 (436)	79.96 (449)	79.88 (486)

Hematocrit at Seven Weeks of Age. In the most recent Report of Progress it was reported with respect to the F_1 generation test groups that differences among treatment group means were significant for neither spermatozoan nor spermatogonial treated series. However, in both cases factorial analyses of variance indicated that the observed differences did approach significance ($p \sim .10$). Analysis of F_2 generation groups revealed highly significant differences among dose group means in the spermatogonial series; differences among dose means in the spermatozoan series, however, were not significant (Table 3). The pattern observed in the F_2 spermatogonial series indicates very little difference between the low dose group and controls but a significant decrease in hematocrit in the high dose group. Inasmuch as other traits (body weight and tail length) of usefulness in detecting mutagenic effects have been of greatest importance in the detection of low dose effects, the hematocrit trait may be of value in detecting the effects of more highly mutagenic agents.

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Table 3. Effect of TEM dose on hematocrit at seven weeks of age of F_1 and F_2 progeny of treated male parents in inbred strain BALB/cBy in Experiment III. Males were treated at the spermatozoan or spermatogonial germ cell stages. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined.

Strain		Stage	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
BALB	F_1	Spermatozoa	$p \sim .11$(n.s.)	48.88 (226)	48.85 (133)	49.28 (72)
BALB	F_2	Spermatozoa	n.s.	48.78 (635)	48.65 (263)	48.90 (97)
BALB	F_1	Spermatogonia	$p \sim .08$(n.s.)	48.73 (365)	48.86 (437)	48.63 (410)
BALB	F_2	Spermatogonia	$p < .005$	48.85 (453)	48.82 (463)	48.51 (507)

The hematocrit analyses exhibit closely parallel response patterns of the F_1 and F_2 generation effects, both for the spermatogonial group and for the spermatozoan group. With respect to the spermatogonial groups the difference between means for the control and low dose group is quite modest in each case and is followed by a much more impressive decrease in hematocrit at the highest dose group. By contrast, in the spermatozoan groups, both the F_1 and F_2 generation means exhibit slight reductions from control to low dose groups followed by modest increases in hematocrit at the highest dose level.

Defecation Portion of the Open Field Test. In the last Report of Progress significant differences were found among dose group means only for the F_1 generation spermatogonial series. However, differences between means in the F_1 spermatozoan series were sufficiently great and the pattern of response among replicates and sexes sufficiently consistent as to suggest that the differences might well be real. In the F_2 generation results no significant differences were found among dose group means for either the spermatozoan or spermatogonial treatment series, but differences between means were appreciable leading to approaches to significance in both cases (Table 4).

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Table 4. Effect of TEM dose on defecation rate at five weeks of age in F_1 and F_2 progeny of treated male parents in inbred strain BALB/cByJ in Experiment III. Males were treated at the spermatoaoan and spermatogonial germ cell stages. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined.

Strain		Stage	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
BALB	F_1	Spermatozoa	$p \sim .20$	4.53 (226)	4.18 (134)	3.92 (72)
BALB	F_2	Spermatozoa	$p \sim .19$	4.17 (641)	4.37 (270)	4.52 (98)
BALB	F_1	Spermatogonia	$p < .05$	4.03 (376)	4.36 (458)	4.38 (417)
BALB	F_2	Spermatogonia	$p \sim .24$	4.70 (462)	4.94 (466)	4.79 (509)

The dose response patterns that emerge are not consistent among the germ cell stage treatment/generation groups, but they are interesting and may well reflect real response patterns. In the F_1 generation spermatozoan groups, defecation rate decreases in a roughly linear fashion with increasing dose. By contrast, in the F_2 generation spermatozoan treatment group, defecation rate increases in a roughly linear fashion with increasing dose. With respect to spermatogonial series treatment, in the F_1 generation there is a pattern involving an increase in defecation rate among treated groups relative to controls, and the differences are significant. However the difference between the two treatment groups is negligible. Finally, in the F_2 generation spermatogonial series, there is a face value increase in defecation rate from the control group to the low dose group followed by a modest decrease.

Brain Weight at Fifteen to Nineteen Weeks of Age. In previous reports we have reported on brain weights at "Approximately Thirteen Weeks of Age". It turns out that this figure is, in practice, much closer to fifteen weeks of age for males and nineteen weeks of age for females. In the last Report of Progress, we provided the results of analysis of brain weights in the F_1 generation spermatogonial group where highly significant differences were found involving an increase in brain weight from control to low dose group followed by a modest decrease in brain weight at the higher dose. Mean values were not provided for the F_1 generation spermatozoan groups because differences among dose groups were not significant. Means for both groups are presented here in Table 5 in order to show the patterns involved.

* * *

Table 5. Effect of TEM dose on brain weight at approximately thirteen weeks of age in F_1 and F_2 progeny of treated male parents in inbred strain BALB/cByJ treated at the spermatozoan and spermatogonial germ cell stages. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined.

Strain	Stage	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
BALB	F_1 Spermatozoa	n.s.	482.11 (212)	481.84 (114)	476.64 (51)
BALB	F_2 Spermatozoa	$p \sim .01$	472.46 (630)	475.72 (266)	473.78 (94)
BALB	F_1 Spermatogonia	$p < .01$	473.04 (350)	476.14 (420)	474.85 (389)
BALB	F_2 Spermatogonia	$p < .001$	464.32 (459)	465.88 (464)	468.29 (506)

It can be seen that the F_1 spermatozoan pattern reflects a very modest drop in brain size from the control to the low dose group followed by a more impressive decrease in brain weight in the high dose group. For both of the F_2 generation groups, differences among dose groups proved to be highly significant, and the patterns are quite interesting. For the F_2 spermatozoan dose groups, there is an increase in brain weight from control to low dose group followed by a modest decrease in the high dose group, the mean brain weight of which is still appreciably greater than control. The relationship of F_2 to F_1 spermatozoan results for brain weight parallels very closely that for body weight in that in both, there are more or less linear decreases in weight (non-significant) with increasing mutagenic dose in F_1 generation results followed by significant increases from control to low dose group

in the F_2 generation results. The parallel continues to high dose groups where brain weight decreased (only slightly) while body weight decreased (appreciably). In the F_2 spermatogonial groups there is a modest increase from control to low dose group followed by an impressive increase to the high dose group. The numbers involved and the improbability that such differences could be due to chance alone leave little doubt as to the reality of these differences.

Results: Dominant Lethality, Experiment IV. Data obtained on dominant lethal parameters for the A and B replicates in Experiment IV are outlined in Table 6. These results parallel quite closely the results obtained in

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Table 6. Dominant lethality parameters with standard errors and sample size of Replicate A and B series BALB/cByJ females and their F_1 young in Experiment IV. Females belonging to the two experimental and a single negative control group were mated for a period of seven days immediately following treatment, thus represent effects of mutagenic agent upon spermatozoa.

	TEM dose, mg/kg of body weight		
	0	.10	.20
Replicate A			
Live Scars/ ♀	4.60 ± 1.12 (20)	3.04 ± .74 (25)	.97 ± .21 (70)
Dead Scars/ ♀	.35 ± .22 (20)	.80 ± .23 (25)	.46 ± .14 (70)
Total Scars/♀	4.95 ± 1.14 (20)	3.84 ± .87 (25)	1.43 ± .30 (70)
Total Live Births/♀	6.73 ± .85 (11)	3.14 ± .63 (14)	1.67 ± .36 (21)
Total Weaned/♀	6.27 ± .92 (11)	3.14 ± .63 (14)	1.43 ± .28 (21)
Percent of Dead Scars of Total	7.1	21.8	32.2
Percent Perinatal Losses	19.6	42.1	48.5
Replicate B			
Live Scars/♀	5.00 ± .94 (19)	2.54 ± .53 (28)	1.84 ± .35 (68)
Dead Scars/♀	.47 ± .19 (19)	.50 ± .17 (28)	.46 ± .11 (68)
Total Scars/♀	5.47 ± .97 (19)	3.04 ± .63 (28)	2.29 ± .41 (68)
Total Live Births/♀	4.87 ± .69 (15)	3.27 ± .40 (15)	2.86 ± .48 (28)
Total Weaned/♀	4.87 ± .69 (15)	3.27 ± .40 (15)	2.79 ± .48 (28)
Percent Dead Scars of Total	8.6	16.4	20.1
Percent Perinatal Losses	23.2	31.0	36.0

Experiment III and demonstrate quite clearly that administration of mutagenic agents was effective. Data on live scars, dead scars and total scars represent numbers per treated female. It can be seen that, as has been found in the past, number of live scars per female decreases more or less linearly with increasing dose as do total scars per female. Dead scars per female,

by contrast, tend to show an increase from control to the lower dose followed by a decrease. Statistical analyses of scar data were carried out by ANOVA; these were based upon numbers of pregnant females (i.e. having uterine scars) within each group. Analysis of total scars was carried out on data after square root transformation. As expected, differences among means due to dose were highly significant ($p < .001$). Differences between replicates were not significant nor were group x dose interactions. A factorial analysis of the percent dead scars of total scars, carried out after arc sine transformation, revealed that the differences among dose groups were significant ($p \sim .014$). Again, there were no significant differences between replicates, and the group x dose interaction was not significant. As has been found in the past, considering only females with scars for this analysis, increase in percent dead scars and in percent perinatal losses from control to both treated groups was impressive, but the difference between treated groups itself was not. It is generally felt that the number of zygotes lost continues to increase with higher doses, but that an increasing proportion of the losses at high mutagenic exposures are made up of pre-implantation losses which are not represented by uterine scars.

Results: Quantitative Traits, Experiment IV. The primary value of this repetition is the demonstration that, as was reported for Experiment III, spermatozoan treated F_1 generation young are not of particular usefulness in the detection of mutagenic activity. This is true with respect to all traits.

Time of Development of the Righting Response. As was the case in Experiment III, no significant differences due to dose were found. Contrary to what was found in Experiment III, there was a roughly linear decrease in the time of development of the righting response with increasing mutagen dose in Experiment IV.

Body Weight at Weaning. Paralleling the results of Experiment III, differences due to dose among F_1 progeny of spermatozoan treated groups were not significant. Parallel elements in the dose means are limited to a decrease in body weight below controls at the highest mutagenic dose in both experiments. However, the approaches to significance are so slight that nothing would be gained by combining the two experiments.

Tail Length at Seven Weeks of Age. The parallel with Experiment III continues with no significant differences among dose groups observed in the F_1 generation spermatozoan treated groups. There is an interesting parallel in that in both experiments, after adjustment for litter size, there is an increase in tail length from control to low dose group followed by a modest decrease. Again, however, approaches to significance in both experiments are so slight as to suggest coincidence in the parallel results.

Hematocrit at Seven Weeks of Age. Again, as was the case in Experiment III, hematocrit differences due to dose were not significant. There was observed an element of similarity in the two experiments in that very slight reductions were found from control to low dose group followed by

an increase of some magnitude in the high dose group. Again, it is estimated that statistical significance would not be achieved by combining the two experiments in a factorial analysis.

Defecation Portion of the Open Field Test. Differences among means approached significance more closely than in other traits in the series ($p \sim .16$), and differences among means in Experiment III also approached significance rather closely ($p \sim .11$). However, the patterns of distribution of means is not similar in the two experiments. There was reported a more or less linear decrease in defecation rate with increasing dose in Experiment III, whereas in Experiment IV there is an increase from controls to both dose groups, with the high dose group mean being slightly higher than the low dose group.

Brain Weight at Fifteen to Nineteen Weeks of Age. As with other traits in F_1 generation spermatozoan test groups, significant differences due to dose were not found in either Experiment III or Experiment IV. Similarity in pattern of mean distribution exists only in that brain weights of treated group progeny were reduced below control means. Differences among means were considerably less in Experiment IV than in Experiment III.

Experiment V: Methods. As outlined in detail in the Report of Progress for the period June 25, 1978 through December 25, 1978, mice of the parental generation were assigned to one of six replicates. The first three of these, carried out two weeks apart, involved mutagenized spermatozoa. These matings involved trios of females mated with each male. The remaining three replicates involved mutagenized spermatogonial cells, the mutagenic treatment having been administered over eight weeks prior to establishment of matings. A schedule of matings was included in the Report of Progress. All matings had been carried out at that time except those for the final replicate, F, which was established on January 8, 1979 instead of January 10, 1979 as planned. In this experiment, in order to maximize number of matings, males were selected from three different age groups and varied from thirteen to nineteen weeks at treatment. Care was taken to assure that all age groups were approximately equally represented in the control and different treatment groups.

A schedule of matings of F_1 generation crosses is provided in Table 7. Pairs and trios of females were mated with single males (in addition to pair matings) in order to use all available females. The numbers of females

* * *

Table 7. Schedule of Matings of F_1 Generation Crosses in Experiment V.

Replicate	A	B	C	D	E	F
Matings Established	2/8/79	2/22/79	3/5/79	2/14/79	3/15/79	5/1/79
Matings Separated	2/22/79	3/8/79	3/19/79	3/28/79	3/29/79	5/15/79

and males involved in F_1 generation crosses are shown in Table 8. Crosses were established between individuals taken randomly within dose and replicate, except that sibling crosses were precluded.

* * *

Table 8. Numbers of females and males involved in F_1 generation crosses in Experiment V. Where females available within a group outnumbered males, pairs or trios of females were mated with males as necessary to exploit all females. Otherwise pair matings were employed.

Replicate:	Spermatozoa Treated			Spermatogonia Treated		
	A	B	C	D	E	F
	♀/♂	♀/♂	♀/♂	♀/♂	♀/♂	♀/♂
control	52/52	52/36	70/62	25/25	46/31	45/38
.1 mg/kg	81/74	85/85	73/73	63/52	51/38	57/23
.2 mg/kg	81/67	96/85	91/69	52/46	40/40	41/35

Results: Dominant Lethality, Experiment V. Three replicates (A, B & C) were employed in Experiment V examining the effects of mutagenic action upon spermatozoa. The data obtained on dominant lethal parameters for these three replicates are outlined in Table 9. In general, these results parallel quite closely those obtained in earlier experiments, particularly those in which strain BALB/cByJ mice have been employed. In Replicate B total scars, which usually decrease from control to the low level dose, actually showed a modest increase. However, the higher dose revealed a substantial reduction in total scars that is characteristic of the dominant lethal test. In Replicate C there was observed a drop in percent dead scars of total from the control to the low mutagen dose. However, dead scars are a low frequency phenomenon, and a modest change can produce a sizable effect upon percentage dead scars of total. Inasmuch as total scars show a very straightforward linear decrease with increasing dose as expected, it seems likely that the low percentage of dead scars in the low dose group is most simply attributable to chance variation. In general, it can be said that live scars and total scars tend to decrease with increasing dose. Dead scars, by contrast, tend to show an increase from control to low dose group. The change from low dose to high dose group is unpredictable. Statistical analysis of dead scars as a percentage of total scars were carried out by ANOVA following arc sine transformation. Differences among dose groups were found to be highly significant ($p \sim .002$). Differences among replicates also proved to be highly significant ($p < .001$), probably due to the aberrant figure recorded for Replicate C described above. Group X dose interactions were not significant. Inspection of the differences between dose group means revealed that the increase in percent dead scars of total was rather modest from control to low dose group, but considerably greater from low dose to high dose group. Analysis of total scars was carried out after square root transformation of data. Differences among dose groups proved to be highly significant ($p < .001$). There was found a clear linear trend of decreasing total scars with increasing mutagen dose in Replicates A and C. While the data of Replicate B were not sufficiently different to affect the statistical results, they probably were responsible for a significant dose x group interaction ($p \sim .024$). It may be recalled that differences

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Table 9. Dominant lethality parameters with standard errors and sample sizes of Replicate A, B and C series BALB/cByJ females and their F_1 young in Experiment V. Females belonging to the two experimental and a single negative control group were mated for a period of seven days immediately following treatment, thus represent effects of mutagenic agent upon spermatozoa.

	TEM dose, mg/kg of body weight		
	0	.10	.20
Replicate A			
Live Scars/♀	5.07 ± .94 (27)	4.15 ± .60 (59)	2.19 ± .30 (112)
Dead Scars/♀	.22 ± .15	.42 ± .18	.66 ± .13 (112)
Total Scars/♀	5.30 ± .97 (27)	4.58 ± .62 (59)	2.85 ± .34 (112)
Total Live Births/♀	7.40 ± .70 (15)	4.91 ± .51 (32)	2.76 ± .38 (55)
Total Weaned/♀	7.00 ± .68 (15)	4.84 ± .37 (32)	2.71 ± .30 (55)
Percent Dead Scars of Total	4.2	9.2	23.2
Percent Perinatal Losses	19.0	35.9	38.0
Replicate B			
Live Scars/♀	3.65 ± .81 (34)	3.64 ± .51 (59)	2.57 ± .33 (115)
Dead Scars/♀	.06 ± .04 (34)	.27 ± .09 (59)	.40 ± .04 (115)
Total Scars/♀	3.71 ± .81 (34)	3.92 ± .54 (59)	2.97 ± .36 (115)
Total Live Births/♀	6.25 ± .70 (16)	5.14 ± .47 (36)	3.92 ± .32 (51)
Total Weaned/♀	5.56 ± .55 (16)	4.83 ± .38 (36)	3.65 ± .28 (51)
Percent Dead Scars of Total	1.6	6.9	13.5
Percent Perinatal Losses	28.2	14.0	32.4
Replicate C			
Live Scars/♀	4.77 ± .74 (44)	3.69 ± .57 (59)	2.59 ± .35 (103)
Dead Scars/♀	.43 ± .15 (44)	.15 ± .05 (59)	.34 ± .09 (103)
Total Scars/♀	5.20 ± .75 (44)	3.85 ± .59 (59)	2.93 ± .37 (103)
Total Live Births/♀	5.81 ± .52 (26)	5.81 ± .49 (27)	3.73 ± .38 (44)
Total Weaned/♀	5.58 ± .45 (26)	5.70 ± .43 (27)	3.41 ± .33 (44)
Percent Dead Scars of Total	8.3	3.9	11.6
Percent Perinatal Losses	28.1	28.0	38.6

among means in total scars due to dose were highly significant in Experiment IV where dead scars decreased in linear fashion with increasing dose in both replicates. Differences among replicates were not significant. In spite of the irregularities present, the analyses of total scars and of

dead scars as a percent of total scars indicate clearly that the mutagenic agent was effective.

Results: Quantitative Traits, Experiment V. I. Spermatozoan Treatment, F_1 Progeny. The results of the analysis of quantitative traits in F_1 progeny of males in which germ cells were mutagenized as spermatozoa are of value for only a few traits. For these traits, differences among means are significant and often they parallel the results found in Experiments III & IV involving the same strain and mutagen doses. It should be born in mind that this experiment involves sample sizes 50% larger than the combined numbers of those involved in Experiments III & IV. In total, data were obtained from nearly 1300 young in the spermatozoan treatment group of Experiment V. Three of the traits involved (body weight at weaning, tail length at seven weeks and hematocrit at seven weeks) would be expected to be of usefulness in detecting mutagenic effects.

Time of Development of the Righting Response. As was found in Experiments III & IV no significant differences due to dose were found. Further, the pattern of distribution of dose means is quite different in Experiment III from the patterns of Experiments IV & V which are strongly parallel. In Experiment V with its relatively large sample size the difference among doses approaches significance ($p \sim .12$). For practical purposes, however, this would not appear to be a very useful trait in the ascertainment of mutagenic damage in F_1 generation young where spermatozoan treatment is involved.

Body Weight at Weaning. In Experiments III & IV differences among dose groups were not significant. However, with the much larger sample size of Experiment V, differences among dose groups are highly significant ($p \sim .005$) after adjustment for litter size (Table 10). There is also a highly significant difference between sexes and among replicates. However, none of the interactions between group, dose and sex is significant.

* * *

Table 10. Effect of TEM dose on body weight at weaning of F_1 progeny of treated male parents in inbred strain BALB/cByJ in Experiments III, IV and V. Males were treated at the spermatozoan germ cell stage. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance. Sexes and replicates are combined for simplicity.

Experiment	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
III	n.s.	18.00 (226)	17.83 (134)	17.67 (72)
IV	n.s.	18.18 (143)	18.27 (97)	18.13 (109)
V	$p < .005$	16.28 (317)	16.66 (488)	16.28 (490)

Inasmuch as there is no difference between means of the control and high dose groups after adjustment for litter size, it follows that the low dose mean is highly significantly greater than both the control and high dose means. The numbers are sufficiently large in this experiment to leave little doubt as to this result. Further, the results of Experiment IV show

the same pattern, that is an increase from control to low dose mean followed by a decrease to high dose mean. However, the differences among means in Experiment IV are of considerably lesser magnitude than are the differences among Experiment V means. In Experiment III there is a drop in body weight from low dose mean to high dose mean that is quite similar in magnitude to that of Experiment IV, but there is, contrary to the results of the other two experiments, a drop in mean body weight from control mean to low dose mean. As was indicated earlier the differences among means in Experiments III & IV did not approach significance. It is emphasized that the discussions above pertain to means adjusted for litter size by covariance. With respect to the raw data, there is a more or less linear increase in body size from control to low dose group followed by virtually no change to high dose group. The maintenance of such high body weight in the high dose group is probably largely attributable to the reduced mean litter sizes with increasing mutagen dose.

As was indicated above, from a purely practical viewpoint, to employ this trait as an indicator of mutagenic activity, experiments should be designed involving multiple replicates and based upon totals of 300 to 500 F_1 young in the control and each test group.

Tail Length at Seven Weeks of Age. As was pointed out above there were close parallels between the results of Experiments III & IV, although differences among dose group means were not significant. Experiment V exhibits the identical pattern and is associated with a highly significant difference ($p < .002$) among dose group means after adjustment for the effects of litter size by covariance (Table 11). Undoubtedly, the large sample sizes (300 to 500 in each group) and an increased magnitude in the difference between means contributed to the statistical significance.

* * *

Table 11. Effect of TEM dose on tail length at seven weeks of age of F_1 progeny of treated male parents in inbred strain BALB/cByJ in Experiments III, IV and V. Males were treated at the spermatozoan germ cell stage. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined for simplicity.

Experiment	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
III	n.s.	82.04 (222)	82.26 (128)	82.07 (70)
IV	n.s.	82.18 (129)	82.51 (95)	82.41 (103)
V	$p < .002$	81.12 (303)	81.75 (472)	81.09 (467)

There is usually a close correlation between body weight and tail length. This correlation is quite clear in Experiments IV & V. If body weights in Experiment III had been closely correlated with tail length (they were not), the parallelism found in Experiment IV & V with regard to body weight would have been even more strongly supported.

After adjustment for litter size, tail length was found to increase from the control to the low dose group, but to decrease to about the level of control in the high dose group; this is the same pattern that was found with respect to body weight at weaning in Experiments IV & V. Returning to tail length in Experiment V, there are highly significant differences among replicates and, as expected, highly significant differences between sexes. However, none of the interactions between replicate, dose and sex was significant.

From the existing evidence, this trait would appear to be most useful in the detection of mutagenic damage in F_1 progeny of males in which germ cells were mutagenized as spermatozoa. In combination with the body weight at weaning trait, reliability of the test would be increased. Nevertheless, substantial numbers (300 to 500) of F_1 young would be required to demonstrate effects with confidence.

Hematocrit at Seven Weeks of Age. The "element of similarity" in respect to hematocrit in Experiments III & IV referred to above are also present in Experiment V. That is to say, there is a reduction in hematocrit from control to low dose group followed by an increase of the same magnitude above the control in the high dose group (Table 12). Thus, the difference between low and high dose group means is about twice as great as the difference between the control and either dose group mean. While the differences among groups are highly significant ($p < .01$), the level of significance is due largely to the magnitude of the difference between treated group means. This trait would be of usefulness in detecting mutagenic damage, particularly in combination with the tail length and body weight traits. In addition to significant differences among replicates and between sexes, there were found highly significant replicate by sex ($p < .002$) and dose x sex ($p < .005$) interactions. Table 12 shows the dose group means after adjustment for litter size for the three experiments considered.

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Table 12. Effect of TEM dose on hematocrit at seven weeks of age of F_1 progeny of treated male parents in inbred strain BALB/cByJ in Experiments III, IV and V. Males were treated at the spermatozoan germ cell stage. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined for simplicity.

Experiment	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
III	$p \sim .11$ (n.s.)	48.88 (226)	48.85 (133)	49.28 (72)
IV	n.s.	48.56 (129)	48.44 (96)	48.70 (104)
V	$p < .01$	47.95 (314)	47.74 (482)	48.13 (483)

Defecation Portion of the Open Field Test. This trait would appear for practical purposes to be of negligible value in the detection of mutagenic effects in F_1 generation progeny of males where mutagenic treatment is directed at spermatozoa. Although all three experiments exhibited differences among means that approached significance ($.1 < p < .2$) the patterns emerging are in striking contrast with one another. As indicated

above, Experiments III & IV were quite different from one another. The results of Experiment V add to the diversity by showing an increase from control to low dose rate like that in Experiment IV followed by a decrease from low to high dose rate comparable to that in Experiment III. It seems reasonable to suppose that some of the differences between means may well be real, but the factors producing the differences need not be those being tested.

Brain Weight at Fifteen to Nineteen Weeks of Age. As in the case of the previous trait, brain weight would not appear to be of practical value in the determination of mutagenic damage involving F_1 progeny of treated males where germ cells are mutagenized at the spermatozoan stage. As in the case of Experiments III & IV no significant differences were found among dose group means. While the patterns of dose group means was parallel in Experiments III & IV, with brain weights of treated groups generally falling below control means, this is reversed in Experiment V where brain weights of treated groups are greater than those of controls.

Results: Quantitative Traits, Experiment V. II. Spermatozoan Treatment, F_2 Progeny. Results obtained in the F_2 generation of spermatozoan treated germ cells are, from a purely practical viewpoint, even less useful than those in the F_1 generation where there were at least a few very exciting traits. There are some very interesting evidences of heritability implicit in some of the results to be discussed below, but the likelihood that this would be a good stage to employ in testing for mutagenicity is not impressive.

Time of Development of the Righting Response. As was found in Experiments III, IV and V in the F_1 generation and in Experiment III in F_2 generation results there were no significant differences among groups. It is worth pointing out a parallel between the results in Experiment III of F_1 and F_2 generation trends. In both, there was observed a more or less linear increase in the time of development of the righting response with increasing dose. Differences among means did not approach significance in the F_1 generation results but did so in the F_2 generation results ($p \sim .10$). It is also of interest that in the spermatogonial F_1 generation results in Experiment III, a similar parallelism exists. This will be discussed below. It should be borne in mind that male parents of the spermatozoan F_1 progeny were also parents of F_1 spermatogonial progeny. However, in one case it would have been the spermatozoan stage cells affected, but in the other spermatogonial cells would have been involved. The similarity of these test results carried out at quite different times is quite provocative.

Body Weight at Weaning. Differences among dose groups in Experiment V were not significant ($p \sim .22$) but they are quite interesting in that they parallel very closely the results of the F_2 generation spermatozoan test group in Experiment III where differences among means were significant ($p \sim .05$). In both groups there is an increase in body weight from control to low dose groups after adjustment for the effects of litter size by covariance. At the higher dose, the results of Experiments III & V are not closely parallel in that there is no difference between the low and

high dose group means in Experiment V, but there is a significant drop from low dose to high dose group mean in Experiment III. The inconsistency of response in high dose means will be seen again in our consideration of spermatogonial groups.

It may be recalled that in the F_1 spermatozoan test groups in Experiment III there was found a non-significant linear decrease in body weight means from control through the low dose to the high dose group that is at variance with other observations with respect to the effect of mutagenic dose on body weight at weaning. It is worth noting that this effect did not appear to be heritable, and it is of even greater interest that before adjustment for litter size there were highly significant increases in body weight in both sexes of both replicates from control to low dose group and to high dose group. Why the adjustments for litter size resulted in such a remarkable difference primarily in the control and high dose group means is difficult to guess, but these data will be examined in greater detail at a later date.

From a practical viewpoint, body weight at weaning in the F_2 generation of the spermatozoan treatment group would not be a good trait to employ in testing for mutagenic effect, but it is about as good as any.

Tail Length at Seven Weeks of Age. In Experiment V, differences among groups were not significant, nor were they significant in the comparable F_2 generation of the spermatozoan treatment group in Experiment III. The only parallel in the results of the two different experiments is that there was a decrease in mean tail length from control to high dose group mean in both experiments, and the decrease is of approximately the same magnitude in each experiment. In the low dose groups, the mean is slightly above control in Experiment III, appreciably below the control mean in Experiment V. It may be recalled that in the F_1 generation spermatozoan treatment groups in all three experiments (III, IV & V) there was an impressive increase from control to low dose group mean followed by a similarly impressive decrease to high dose group mean. However, differences among tail length means were not significant except in the case of Experiment V ($p < .002$). Thus, it might be said that the pattern in the F_2 generation spermatozoan treatment in Experiment III is reminiscent of the F_1 generation spermatozoan test group results except that the increase from control to low dose group mean is very slight, while the subsequent decrease is several times as great. In Experiment V, it is of interest that the usually observed strong correlation between body weight and tail length is not evident. As with the body weight trait, it may be said that tail length is not a good trait to test for mutagenicity in the spermatozoan treatment stage in the F_2 generation.

Hematocrit at Seven Weeks of Age. This trait is probably the most interesting in this particular germ cell stage test group because of the parallels that exist with the same germ cell stage in Experiment III and with Experiments III, IV & V in the F_1 generation spermatozoan treatment groups.

In Experiment V differences among groups approach significance ($p \sim .055$). The most important element in common in all five of the sets of results cited above is a drop from control to low dose group. In all experiments except V, there is a face value increase from low dose group to high dose group mean, not significant in Experiment III, F_2 spermatozoan test group, but approaching significance in Experiment III, F_1 spermatozoan test group ($p \sim .11$). In the F_1 generation spermatozoan test group in Experiment IV the increase was not significant, but it was highly significant in Experiment V ($p < .01$).

While these evidences go a long way toward establishing the reality of the differences dealt with, the numbers required to demonstrate a significant effect would not lead one to conclude that this is a useful trait for the ascertainment of mutagenic effect in an F_2 generation spermatozoan treated test group.

Defecation Portion of the Open Field Test. Differences among groups in Experiment V are not significant. From an academic viewpoint it is of interest that there is a face value increase from control to low dose group followed by a decrease from low to high dose group mean as was found in the F_1 generation spermatozoan test group in Experiment V. In the F_2 generation spermatozoan test group, Experiments III & V have in common only an increase from control to low dose group means. In general, it may be said that none of the spermatozoan groups tested for this test in either experiment has revealed significant differences among dose group means.

Brain Weight at Fifteen to Nineteen Weeks of Age. In Experiment V differences among means are not significant, and the distribution of means in Experiments III & V are in remarkable contrast to one another. In Experiment III differences among means proved to be highly significant based upon increases in both treated groups over the control mean (see Table 5). In the Experiment V results, there is an interesting parallel between F_2 spermatozoan and F_1 spermatozoan treatment groups in that there is a decrease from control values to both low and high dose group means, but in neither case were there significant differences among means.

It is clear that brain weight would not be a useful trait for the ascertainment of mutagenic damage in F_2 generation spermatozoan treated groups.

In summary, if it is desirable to ascertain with confidence the heritability of mutagenic effects by testing an F_2 generation, spermatozoan treatment would not be recommended employing BALB/cByJ as an experimental strain.

Results: Quantitative Traits, Experiment V. III. Spermatogonial Treatment, F_1 Progeny. In Experiment V, in spermatogonial treatment groups, significant differences due to dose were found with respect to every trait examined with the exception of brain weight. As indicated above in Experiment IV, there was no comparable treatment group. However, there were reported above results from a comparable group in Experiment III, and

comparisons will be made in the discussion below with respect to specific traits. It may be recalled that significant differences among dose groups were found in all except two traits in Experiment III. These were time of development of the righting response and hematocrit. With respect to three traits, significant differences among dose groups were found in both Experiments III & V, and the dose mean differences that are significant in both experiments are parallel. It is of particular interest that these parallel differences were found with respect to the control and low dose treatment group means. These three traits are: body weight at weaning, tail length at seven weeks and defecation portion of the open field test.

Time of Development of the Righting Response. In Experiment III with sample sizes exceeding 1200, no significant differences were observed among dose groups as reported above. In contrast, in Experiment V with less impressive sample sizes totaling some 750 young, highly significant differences were found among dose groups ($p < .01$) after adjustment for litter size by covariance. There was observed a linear decrease in the time of development (increase in rate) of the righting response with increase in mutagen dose. There were also highly significant differences ($p < .001$) among the three replicates and a significant group x dose interaction ($p < .05$). In Experiment III there were also highly significant differences ($p < .001$) between replicate means but no significant interactions or other complications.

It can only be concluded that factors other than those being tested may have major effect upon the time of development of the righting response. In general, it may be said that all of the three replicates in Experiment V exhibited greater mean righting response times than those observed in Experiment III. Of course, changes in food or water quality, or subtle environmental changes of which we are not aware could be responsible for the general differences between times of development of the righting response or the differences in response to mutagenic agent observed in the two experiments. It seems evident that until such factors can be identified and controlled, this trait will not be particularly reliable for the detection of mutagenic effects in F_1 generation young of males treated at the spermatogonial germ cell stage. An equally evident conclusion is that the time of development of the righting response may be affected by mutagenic treatment. Interestingly, the highly significant decrease in time of development of the righting response with increasing mutagen dose parallels closely the results of the F_1 generation spermatozoan test groups ($p \sim .12$), but not those of the F_2 spermatozoan groups.

Body Weight at Weaning. In Experiment III it may be recalled that highly significant differences among dose group means ($p < .001$) were found in this treatment stage after adjustment for litter size by covariance. These differences were based upon a roughly linear increase in body size with increasing mutagen dose. In Experiment V significant differences were also found among dose group means ($p < .025$) after adjustment for litter size (Table 13). This statistical significance is due primarily to an increase in mean from control to low dose group that parallels very closely the difference between control and low dose group in Experiment III.

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Table 13. Effect of TEM dose on body weight at weaning of F_1 and F_2 progeny of treated male parents in inbred strain BALB/cByJ in Experiments III and V. Males were treated at the spermatogonial germ cell stage. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance. Sexes and replicates are combined for simplicity.

Experiment	Generation	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
III	F_1	$p < .001$	16.49 (376)	16.87 (458)	17.06 (419)
V	F_1	$p \sim .025$	15.53 (221)	16.03 (287)	15.81 (258)
III	F_2	$p < .01$	16.84 (462)	17.17 (466)	17.11 (509)
V	F_2	$p < .05$	15.78 (515)	15.86 (617)	15.56 (581)

In contrast to the situation in Experiment III, there is a decrease in body weight between the low and high dose group means which, however, is not significant. Similarly the difference between low and high dose means in Experiment III is not significant. Thus, the general conclusion that may be drawn from these two experiments, III and V, is that body weight at weaning, adjusted for litter size by covariance, increases with mutagenic treatment. However, the effect of doubling the mutagenic dose while not entirely clear, appears to be other than linear.

It seems likely that this trait, particularly in combination with that to be discussed next, tail length at seven weeks of age, could be quite useful in providing a very sensitive method for the detection of mutagenic damage in spermatogonia. This is particularly important when it is considered that very few mammalian tests are effective in the detection of spermatogonial damage. Since it is in spermatogonial cells that mutagenic damage may accumulate, the usefulness of this assay could be very great. Cytogenic techniques may also be employed to detect mutagenic damage in spermatogonial cells. Whether this assay is comparable in sensitivity to such techniques can only be determined by direct comparison, but if the effects we have measured are based upon polygenic variation as seems most probable, sensitivity of this assay could prove to be very great indeed.

Tail Length at Seven Weeks of Age. The parallel between the results of Experiments III & V are impressive. After adjustment for the effects of litter size by covariance, differences among means based upon factorial ANOVA were highly significant ($p < .005$) in Experiment III. In Experiment V, differences among means after comparable analysis were also highly significant, ($p < .001$). In both experiments there is a parallel increase in magnitude from control to low dose group mean followed by a decrease of considerably lesser magnitude in high dose group means (Table 14). The similarity in the distribution of tail length means and the means for body weight is quite clear, and this has been observed on a number of occasions in other experiments involving the same and other inbred strains. In this particular case, tail length seems to have provided a more reliable measure of mutagenic effect than body weight at weaning. However, the correlated results of both traits provide an indication of even greater reliability.

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Table 14. Effect of TEM dose on tail length at seven weeks of age of F_1 and F_2 progeny of treated male parents in inbred strain BALB/cByJ in Experiments III and V. Males were treated at the spermatogonial germ cell stage. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined for simplicity.

Experiment	Generation	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
III	F_1	$p < .001$	81.88 (366)	82.57 (441)	82.51 (406)
V	F_1	$p < .005$	79.64 (217)	80.47 (262)	79.93 (238)
III	F_2	$p < .05$	79.60 (436)	79.96 (449)	78.89 (486)
V	F_2	n.s.	81.72 (500)	81.85 (583)	81.75 (535)

In both Experiments III and V, differences between the sexes with respect to tail length are, as usual, highly significant ($p < .001$) and differences between replicates (Experiment III) and among replicates (Experiment V) are significant. In both experiments there is an interesting indication of a dose x sex interaction, significant in Experiment III ($p \sim .02$), but only approaching significance in Experiment V ($p < .10$). As with body weight at weaning the effect of doubling the mutagenic dose is not to produce more of the effect of a single dose. It appears that tail length, adjusted for litter size by covariance, increases with mutagenic treatment. However, the effect of increasing mutagenic doses is other than linear and may actually result in a reversal of direction of effect. This has been observed many times with a number of traits, and it seems quite likely that it is real.

Hematocrit at Seven Weeks of Age. The similarities that exist between Experiments III & V with respect to hematocrit are not based upon differences that are significant. However, the observed increase in hematocrit from control to low dose group mean may very well be real. In Experiment III, differences among groups only approached significance ($p < .10$), but the greatest difference was that between the two dose group means. In Experiment V, there are highly significant differences among groups ($p < .001$), but both low and high dose group means are greater than the control group mean, and there appears to be a roughly linear increase in hematocrit with increasing dose (Table 15).

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Table 15. Effect of TEM dose on hematocrit at seven weeks of age of F_1 progeny of treated male parents in inbred strain BALB/cByJ in Experiments III and V. Males were treated at the spermatogonial germ cell stage. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined for simplicity.

Experiment	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
III	$p < .10$ (n.s.)	48.73 (365)	48.86 (437)	48.63 (410)
V	$p < .001$	48.08 (224)	48.32 (285)	49.04 (256)

In view of the limited response, if real, of hematocrit to low mutagen doses and the variable response to high dose treatment in this germ cell stage, hematocrit would not seem to be a very likely prospect for the efficient ascertainment of mutagenic damage.

Defecation Portion of the Open Field Test. Next to the tail length and body weight traits, defecation rate exhibited the most interesting parallels of any in the spermatogonial F_1 generation results (Table 16). In both experiments (III and V) differences among means were significant ($.01 < p < .05$). Although there did not appear to be significant variation due to litter size, the factorial ANOVA was carried out after adjustment for whatever litter size effects existed. In both experiments there were highly significant differences between sexes. In Experiment III there was no significant difference between replicates, but in Experiment V differences among replicates were highly significant. In neither experiment were any interactions significant.

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Table 16. Effect of TEM dose on defecation rate at five weeks of age in F_1 and F_2 progeny of treated male parents in inbred strain BALB/cByJ in Experiments III and V. Males were treated at the spermatogonial germ cell stage. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined.

Experiment	Generation	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
III	F_1	$p < .05$	4.03 (376)	4.36 (458)	4.38 (417)
V	F_1	$p \sim .01$	4.10 (229)	4.38 (299)	3.84 (258)
III	F_2	n.s.	4.70 (462)	4.94 (466)	4.79 (509)
V	F_2	n.s.	3.74 (521)	3.83 (619)	3.74 (581)

As with the body weight and tail length traits, there are important parallels between the control and low dose group means. Also as with the body weight and tail length traits, the low dose and high dose group relationships are dissimilar. It is of interest that there is a highly significant drop from low dose group to high dose group in Experiment V which parallels the face value change of the body weight and tail length traits in the same experiment. In contrast, in Experiment III, the difference between low and high dose group means is not significant, and varies in the three traits from slight increases in fecal deposition rate and body weight to a slight decrease in tail length. In view of the close parallel of defecation rate, body weight and tail length traits, it is reasonable to suggest that all three traits employed together would provide an element of reliability of results that could be most useful.

Brain Weight at Fifteen to Nineteen Weeks of Age. This trait, which appeared to have interesting potential in work with other strains and in the same germ cell stage treatment group with BALB/cByJ in Experiment III, failed to show significant differences among means in Experiment V (Table 17). In contrast with the highly significant increase from control to

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Table 17. Effect of TEM dose on brain weight at approximately fifteen (males) to nineteen (females) weeks of age in F_1 progeny of male parents in inbred strain BALB/cByJ treated at the spermatogonial germ cell stage. Results of Experiments III and V are included. Sample sizes are given in parentheses. Means have been adjusted for the effects of litter size by covariance, and sexes and replicates are combined.

Experiment	Generation	Significance	0 mg/kg	0.1 mg/kg	0.2 mg/kg
III	F_1	$p < .01$	473.04 (350)	476.14 (420)	474.85 (389)
V	F_1	n.s.	457.05 (210)	455.66 (288)	455.68 (251)
III	F_2	$p < .001$	464.32 (459)	465.88 (464)	468.29 (506)
V	F_2	$p < .001$	442.22 (506)	444.87 (607)	447.69 (551)

low dose group mean in Experiment III, there was a face value decrease from control to low dose group mean in Experiment V. From these data it is clear that a consistent effect cannot be expected, and brain weight would not be a trait of choice employing strain BALB/cByJ in an F_1 generation test of spermatogonial damage.

Results: Quantitative Traits, Experiment V. IV. Spermatogonial Treatment, F_2 Progeny. In general, in spermatogonial test groups, the results of tests involving F_2 progeny are almost as useful and quite as exciting as those involving F_1 progeny. Tests involving spermatozoan test groups, either F_1 or F_2 generation, have been far less interesting. In these latter groups, except for three traits (body weight at weaning, tail length at seven weeks and hematocrit at seven weeks, and these only in the F_1 spermatozoan test groups), there have really been few traits that would appear to be of potential usefulness in the practical ascertainment of mutagenic damage. However, in striking contrast, most traits exhibited significant differences among dose groups in one or the other experiment in F_1 spermatogonial test groups, and three, body weight at weaning, tail length at seven weeks and defecation rate showed parallel effects in pattern in Experiments III and V, primarily with respect to differences between control and low mutagen dose groups. With respect to F_2 generation spermatogonial tests Experiment III results were discussed above. In experiment V, three traits stand out in exhibiting, as in Experiment III, significant differences among means and in having parallels with the mean distributions of Experiment III. These are, perhaps curiously, body weight at weaning, hematocrit at seven weeks and brain weight at fifteen to nineteen weeks of age. But, perhaps equally important, the defecation rate and tail length traits, which showed such impressive effects of mutagenic treatment in F_1 spermatogonial test groups, offer strong support for those conclusions by demonstrating very similar mean distribution patterns in F_2 results. The lack of significant differences seems largely a result of decreased magnitude of the differences between means. It will be attempted in this section to tie together meaningful comparisons not only of Experiments III and V, but also meaningful comparisons of F_1 and F_2 spermatogonial results.

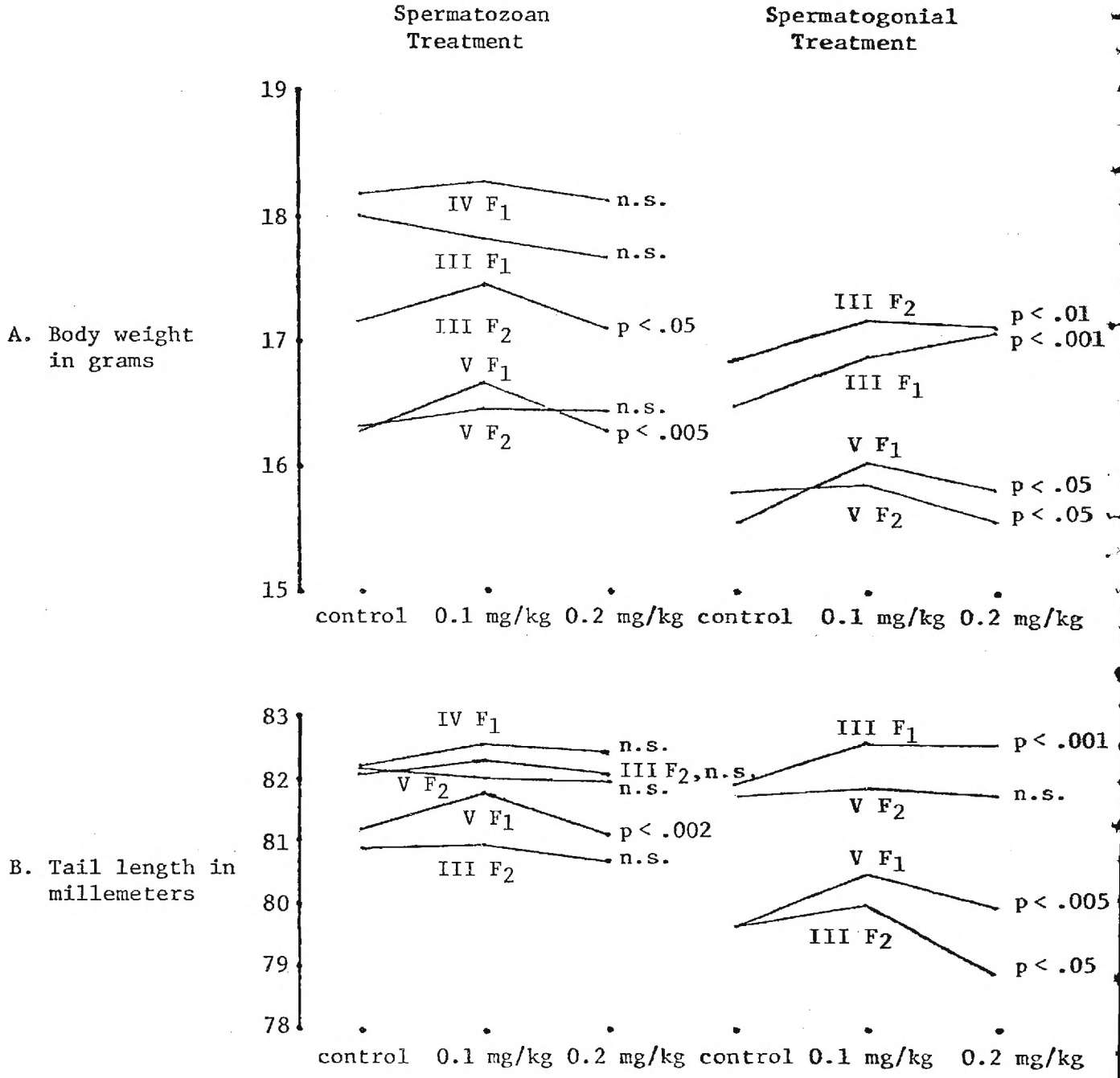
Time of Development of the Righting Response. In Experiment V, there were not found significant differences among means and, in fact, such differences were of lesser magnitude than in any other germ cell stage/generation group tested. It may be recalled that there were highly significant differences among means in the F_1 generation spermatogonial tests based upon a roughly linear decrease in time of the development (increase in developmental rate) of the righting response with increasing mutagen dose. There seems to be little doubt as to the reality of the differences found in that experiment, but either the responsible genetic variance was lost, or the appropriate environment to foster the development of the differences observed did not exist, when the F_2 generation experiments were conducted, or both. Further, F_1 spermatogonial test results from Experiment III, reported above, did not support the F_1 spermatogonial results of Experiment V, and the F_2 generation results in Experiment III only support those of Experiment V in exhibiting very modest differences among means and no particular pattern of response.

The righting response trait, at least insofar as strain BALB/cByJ is concerned, is not a very useful trait to employ in the ascertainment of mutagenic damage.

Body Weight at Weaning. In Experiment V differences among means were found to be significant as they were in Experiment III in spermatogonial, F_2 generation tests (Table 13). It will be recalled that there were also significant differences among means in both Experiments III & V in the F_1 generation spermatogonial test groups. However, the primary importance of the F_2 spermatogonial generation results does not lie in their usefulness for the ascertainment of genetic damage, but in the evidence they provide for heritability of effect. The F_1 generation spermatogonial stage tests would serve far better for detection of mutagenic effects. The mean distribution patterns seen in the F_2 tests are nicely derivable genetically from those found in the F_1 tests. There are increases in the F_2 generation tests from control to low dose group in both experiments. This difference is not significant in Experiment V, although it is in Experiment III. In the F_1 generation spermatogonial tests, the increase from control to low dose group was impressive and significant in both experiments. In Experiment V, F_1 generation results, there was evident an appreciable drop from low to high dose group, and this same trend is present in the F_2 spermatogonial results and the difference is significant. One interesting difference: the high dose group in Experiment V F_1 was appreciably above control, while in the F_2 results the same group is appreciably below control. However, there is a decrease from low dose mean to high dose mean in both F_1 and F_2 generation results. The relationship between the high dose means of Experiment III & V remains approximately the same in the F_1 and F_2 generation results (see Figure 1A).

These patterns just described have close similarities to mean distribution patterns for spermatozoan results in both the F_1 and F_2 generation with the notable exception that, in Experiment III of the F_1 generation, there was an apparent linear decrease in body weight with increasing mutagen dose. However, differences among means in this experiment were not significant.

Figure 1. Effect of triethylenemelamine dose on body weight at weaning and tail length at seven weeks in F_1 and F_2 generation progeny of treated males of inbred strain BALB/cByJ in Experiments III, IV and V. Effects of treatment on spermatozoa and spermatogonia are shown. Sexes and replicates are combined for simplicity. Probability figures shown are taken from factorial ANOVA results for each experiment and refer to significance of differences among means within an experiment.



Tail Length at Seven Weeks of Age. Differences among means due to dose in Experiment V, F_2 generation spermatogonial results were not significant, but the pattern of distribution of means is very similar to what it was in the F_1 generation spermatogonial results as well as to the results of Experiment III in both F_1 and F_2 generation spermatogonial tests. This pattern involves an increase from control to low dose group followed by a less pronounced decrease to the high dose group. In all spermatogonial tests except this, significant differences among tail length means were recorded. These results are important in that they provide evidence of heritability with the magnitude of the differences among means reduced in F_2 generation results. In the F_2 spermatogonial results of Experiment III, the mean distribution pattern is similar to that in Experiment V, and the magnitude of the differences among means are reduced, but the increase from control to low dose group is significant (Table 14).

Only one of the spermatozoan tests, that for the F_1 generation in Experiment V, resulted in significant differences among means, and these were impressively so. In this test, there was also found the generally observed increase from control to low dose group mean followed by a decrease to the high dose group mean. The distribution of means for all tests, spermatogonial and spermatozoan, are graphed for ease of visualization in Figure 1B.

Hematocrit at Seven Weeks of Age. In Experiment V, differences among dose group means were significant, with the significance itself due primarily to an increase from control to low dose group in the F_2 spermatogonial results. In F_1 generation spermatogonial results of the same experiment there were highly significant differences among means and an increase from control to low dose group comparable in magnitude to that in the F_2 results.

With respect to the response in the high dose group, results are quite dissimilar in the F_1 and F_2 generations. In the former, there was a highly significant increase from low to high dose group; in the F_2 generation results there was a decrease. As has been pointed out previously, high dose group results do appear to be erratic and unpredictable.

Elements of concordance between the results of Experiment III and V are present, but not very useful. In F_1 generation results of Experiment III there was found the same increase from control to low dose group that was found in Experiment V. However, unlike the results of Experiment V, there was a non-significant decrease from low dose to high dose group means. In F_2 generation results, by contrast, Experiments III and V are alike in exhibiting decreases from low to high dose group means. However, in Experiment III there was recorded a slight decrease from control to low dose group mean as opposed to the significant increase found in Experiment V.

Everything considered, while there may be elements of similarity based upon heritability, hematocrit, in strain BALB/cByJ, would not appear to be useful either in providing persuasive evidence of heritability of mutagen induced variance or, for practical purposes, in the ascertainment of genetic damage. Even so, the impressive levels of significance involved in certain differences do indicate that effects of mutagenic treatment exist, but they appear to be unpredictable.

Defecation Portion of the Open Field Test. The results of Experiment V in the F_2 generation of the spermatogonial test are quite comparable to those of Experiment III not only in that there are no significant differences between means, but also in the close parallel in distribution of means. In both experiments there is an increase from control to low dose mean followed by a decrease from low to high dose mean. As has been noted with respect to other traits, this is the same pattern found in F_1 generation spermatogonial results (with the exception of one data point) except that the differences between F_2 generation means are much reduced. Inasmuch as the differences among means in the F_1 results were significant in both experiments III and V, it is reasonable to suppose that the effects are heritable but with reduced degree of expression in the F_2 generation.

For purely practical purposes the defecation rate trait would not appear to be of usefulness in an F_2 generation spermatogonial test to ascertain mutagenic damage.

Brain Weight at Fifteen to Nineteen Weeks of Age. Results obtained with this trait are clearly the most useful of those obtained in the F_2 generation of the spermatogonial tests. As with the results of Experiment III, there are highly significant differences among means ($p < .001$), and the results of Experiment V parallel precisely those of Experiment III in that there is a linear increase from control to low dose group continuing on to high dose group. The magnitude of the increases in both experiments are quite comparable (Table 17).

It is concluded that brain weight can provide a very sensitive measure of mutations induced at multiple loci by a mutagenic agent in the F_2 generation progeny of treated males. It should be pointed out that results with F_1 young may have been distorted to an appreciable extent by the necessity of permitting females to rear their young to weaning before taking the brain weight of the females themselves. This introduced a variable that may be partioned out by a more detailed analysis. Most importantly, however, this factor does not intrude into the measurement of brain weight of the F_2 progeny because there was no intention to produce an F_3 generation and no need to distribute brain weight measurements over an excessively broad span of ages.

Discussion. The purpose of these experiments has to determine the usefulness of the cumulative effects of mutations induced by a powerful mutagenic agent, triethylenemelamine, in bringing about changes in means of a series of traits of a continuously varying nature and, therefore, likely determined by genes at multiple loci.

Dominant lethal effects have been studied in the F_1 generation of every experiment conducted primarily to assure that the mutagenic agent employed had been effective, and also to provide a standard by which any polygenic effects observed might be compared. Suffice it to say that in every experiment conducted, highly significant dominant lethal effects were observed, thus indicating that potent mutagenic agent had been successfully administered. These experiments have led to the confirmation of

a hitherto undescribed late dominant lethal effect, the results of which have been published (Favor, Soares and Crenshaw, Mutation Research, 54 (1978) 333-342). This dominant lethal effect was found to occur at about the time of parturition and to vary directly with increase in mutagen dose.

In the discussion of specific effects below, we have concentrated upon a single mouse inbred strain, BALB/cByJ, primarily because the last three experiments were conducted with this strain because of its very high productivity. Some of the other strains studied probably exhibited much greater mutagenic effects, but the poor productivity of the strains could make it considerably more expensive to conduct experiments of the size required to demonstrate mutagenic effect. In fact, it is not known that the economics of the situation would favor use of the BALB strain employed, but indications from preliminary studies made it seem most likely that this would be the case. Nevertheless, the other strains studied, Strain C3H/HeJ and DBA/2J, showed clear indications of response, some similar, some different, to the same mutation dose and employing the same traits.

One generalization may be stated that may be of some usefulness with respect to sample sizes required to demonstrate statistical significance in most groups in these studies. In general, in order to provide a base sufficient for useful statistical analysis of the data developed for the traits recommended below, on the order of 400 to 500 young will be required for each control and experimental group in an experiment. These numbers may be produced by combining two or three replicates, but the replicates should be carried out in reasonably close temporal succession.

Although it has not been specifically stated in the discussion of every trait in the report above, the statistical analyses carried out are always factorial analyses of variance (dose x replicate x sex). In every case, dose/group means have been adjusted by covariance for the effects of litter size, even if the effects of litter size could not be shown to be significant.

In view of the goal of these experiments, it has been far more important to demonstrate repeatable effects than to demonstrate rectilinear relationship between increasing mutagenic dose and degree of effect. An interesting fact that has emerged, that appears to be repeatable and that appears from a number of evidences to be heritable, is variation in the direction of response with the two different doses employed. While rectilinear response was observed in some traits, quite often traits were found to increase above control at the low dose level and to decrease back toward or beyond control level at the high dose. The opposite was also observed for other traits, that is to say decreased response occurred at the low dose level relative to control, followed by a return toward or even above control level at the high dose. It does not strain the imagination to develop hypotheses to account for this sort of response. For the body weight trait, for example, it is reasonable to suppose that a heterotic effect may obtain at low mutagen dose, or simply that allelic mutants for increased body size tend to be dominant over those for decreased body size. Either mechanism could account for increases in body size at low mutagen doses. However, it is clear that if enough mutations are induced, as by a doubled mutagenic

dose, the net effect on the development of the organism may be sufficiently detrimental as to lead to a general reduction in body size or failure to develop the same body size at the time weights are taken.

* * *

Turning to a specific consideration of the traits of importance in each generation of the two germ cell stages tested, we will consider first F_1 generation responses in those tests in which germ cells were treated as spermatozoa. It may be recalled that only three traits proved to be potentially useful in reflecting effects of the mutagenic agent. However, the economic significance of this stage is very great inasmuch as experiments can be run in a relatively short time, about six months. (F_1 spermatogonial tests would require an additional 2 months and F_2 spermatozoan tests require approximately a year.)

In Experiment V, with over 300 young involved in the control group and between 450 and 500 in each of the experimental groups, there were highly significant differences among dose groups with respect to both body weight and tail length. In both traits there was an increase from control to low dose group followed by a decrease to high dose group. With respect to both traits the low dose mean was highly significantly greater than either the control or the high dose mean. Accordingly, it is suggested that these two traits, in combination, would be quite useful in the detection of the effects of agents of relatively low mutagenicity. With respect to the same germ cell/generation test in Experiments III and IV, differences among means were not significant, in part because of relatively low numbers. (Both experiments combined included less than 1300 mice.) However, the distribution of means in both experiments follows the same pattern as that in Experiment V with respect to tail length, and the pattern of means in Experiment IV paralleled that in Experiment V with respect to body weight.

Hematocrit also proved to be useful in Experiment V as an indicator of mutagenic damage. In this case, there was a modest reduction (non-significant) from control to low dose treatment group followed by an appreciable increase (highly significant) between low and high dose group means. Although differences among means were not significant for the comparable germ cell stage/generation tests in Experiments III and IV, the pattern of distribution of means offers precise support for the results of Experiment V with its much larger numbers. It is our interpretation, that hematocrit might be particularly useful in the ascertainment of the effects of agents of relatively high mutagenicity. In every case the reduction from control to low dose was relatively modest, and the subsequent increase to high level dose mean somewhat greater.

We conclude that the traits body weight at weaning, tail length at seven weeks and hematocrit at seven weeks may prove to be quite useful in the detection of mutagenic effects over a broad range of dosages, employing the strain BALB/cByJ in adequate numbers, in an F_1 generation test involving mutagenized spermatozoa.

* * *

Results of the analysis of data on F_2 generation young in the spermatozoan treated series indicate that this is not a particularly useful stage for in the detection of mutagenic effects.

With respect to body weight, evidence of heritability of the increase from control to low dose group followed by a decrease to high dose group was found in Experiment III, and the differences among means were significant ($p < .05$), in spite of relatively modest numbers. (There were approximately 650 young in the control group, 270 in the low dose and 100 in the high dose group.) That numbers are not the problem, however, is indicated by the results of Experiment V in which about 750 young were tabulated each for control and high dose group and over 1000 for the low dose group. In this experiment, heritability of the F_1 generation effect of increase from control to low dose group was indicated, but there was no subsequent decrease to the high dose group as in the F_1 generation. None of these differences however were significant in Experiment V.

With respect to the hematocrit trait, heritability of effect was suggested by the results of both Experiments III and V in that there was a drop from control to low dose group paralleling that of the F_1 generation results. In Experiment V, with the very large numbers involved, this difference approached significance ($p \sim .055$). Also paralleling the F_1 results, there was an increase from low dose group to high dose group in Experiment III (not significant) but not in Experiment V.

In spite of the often inconclusive results of analyses of our data on brain weight, the impressive results to be discussed below with respect to spermatogonial effects suggests that prospects for this trait may be explored a bit further before being discarded as a trait suitable for spermatozoan testing. In the first place, there has been a problem of variation in time of acquisition of brain weight due, in females, to the necessity of permitting F_1 females to rear their young to weaning prior to taking brain weights. In males also, in F_1 generation results, brain weights have been postponed for some males while tests for evidence of translocations were carried out. More detailed analyses of our data will be carried out with appropriate partitioning of these variables as time permits. In our F_2 generation spermatozoan germ cell stage test, in Experiment III, there was a highly significant increase in brain weight from control to low dose group, paralleling a significant increase in body weight in the same experiment (there was also a non-significant decrease from low to high dose group, also paralleling a decrease in body weight). However, this mean distribution pattern received no support from the results of Experiment V in which very large numbers were involved. It is worth noting, however, that the F_2 generation spermatozoan results in Experiment V with respect to brain weight showed interesting similarities, suggesting heritability, to the F_1 generation spermatozoan results. Clearly, at this stage of development, brain weight would not appear to be a useful trait for the detection of mutagenic damage in spermatozoan test groups.

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The results of both of the spermatogonial generation tests are exciting, and show considerable promise for use in the detection of mutagenic activity. On the one hand, it is of particular interest that mutations induced in spermatogonial cells are relatively easily detectable, because there are not many tests available in mammalian systems in which spermatogonial damage can be detected. The question as to why this might be so in our test when spermatozoan damage is not so easily detected is an interesting one. It is suggested that for most of the traits employed, it may be that mutations induced in spermatozoa may be so much more dramatic in their effect in both directions that erratic results eliminate the general trends which may be evident in spermatogonial damage, where far less dramatic mutations only may survive repair or elimination.

The F_1 generation spermatogonial germ cell stage results were far and away the most exciting and useful of any obtained. With respect to three traits, body weight at weaning, tail length at seven weeks and the defecation portion of the open field test, significant differences were found to exist among means in both Experiments III and V, and the dose mean differences that were significant in both experiments were parallel.

With respect to body weight at weaning, there were increases in body weight from control to low dose groups in both Experiments III and V, highly significant in the former, significant in the latter. In both experiments, differences between low and high dose groups are not significant. In Experiment III there was recorded a face value increase; in Experiment V, a decrease.

Closely paralleling these body weight patterns, there were highly significant increases in tail length in both Experiments III and V between the control and low dose groups. In both experiments there were decreases from low dose to high dose groups, but these differences were not significant. It is of particular interest that the numbers involved in this particular germ cell stage/generation test were relatively modest. In Experiment III, numbers ranged from about 370 in the control group to between 360 and 410 in the experimental test groups. In Experiment V, the numbers were even lower, ranging from about 220 in the control groups to between 240 to 260 in the experimental groups.

Employing the traits body weight at weaning and tail length at seven weeks in a spermatozoan and a spermatogonial, F_1 generation test, there exists in the approach under investigation here a powerful mechanism for the demonstration of mutagenic effects, particularly with relatively low potency mutagenic agents.

The defecation portion of the open field test also revealed significant increases from control to low dose group, paralleling the body weight and tail length traits, in both Experiments III and V. While the levels of significance in these experiments were not as impressive ($.01 < p < .05$) as in the body weight and tail length traits, incorporation of this trait would add an additional degree of confidence to an F_1 spermatogonial test.

The parallelism with the body weight and tail length traits extends to the relationship between low dose and high dose group means, but in an interestingly different way. In Experiment V with respect to all three traits, body weight, tail length and fecal rate, there was observed a decrease from low dose to high dose group. In Experiment III some similarity was found in that there is little difference between low and high dose group means for all three traits. In body weight there is a modest increase from low to high dose; in tail length there was a modest decrease from low to high, and in fecal rate there was very little difference.

* * *

As indicated above, the F_2 generation spermatogonial germ cell stage test was not nearly so impressive in the kinds of conclusions it provided as were the F_1 generation spermatogonial tests. This was true in spite of considerably greater numbers (450 to 600 in most test groups). However, some of the most interesting results with respect to heritability were developed in this group, and brain weight proved to be a remarkably exciting and useful trait in contrast to what it had been in other groups.

With respect to the brain weight trait, there proved to be a highly significant more or less linear increase in brain weight with increasing mutagen dose in both Experiments III and V, and the mean distribution patterns are quite similar in both experiments. It is probably not just coincidental that this was an F_2 generation test and one in which brain weights could be taken over a relatively short span of time in the life of the experimental animal. Everything considered, this germ cell stage/generation group would not be one of choice if the primary interest were the confident ascertainment of mutagenic damage. However, brain weight stands out as the best choice of traits if it were desirable, on other grounds, to test mutagenic effect in the F_2 generation of mutagen induced spermatogonial damage. It seems likely that spermatogonial mutagenesis generally results in fewer unrepaired mutations as well as mutations of a less dramatic and extensive nature than is the case in spermatozoan mutagenesis. It further seems likely that even this damage may be somewhat diluted by selection in transmission from the F_1 to the F_2 generation. Accordingly, it is concluded that very slight increases (with increases in mutagenic dose) in the frequency of mutations (themselves of very slight effect) results in an increase in brain weight in strain BALB/cByJ. It is reasonable to suppose that the reduction in brain weight size often observed (but not as a significant effect in our experiments) may be real and may result from an increase in the frequency of mutations generally or in the frequency of mutations having a generally deleterious effect upon brain development.

The hematocrit trait in F_2 spermatogonial results was of interest in that there were differences among means in both Experiments III and V (respectively $p < .005$ and $p < .05$). However, the parallel aspect of the mean distributions lies only in a reduction from low to high dose group. This difference is means is highly significant in Experiment III, but not in Experiment V. There was an impressive increase in Experiment V from control to low dose group but little difference between control and low dose group in Experiment III.

One other trait, body weight at weaning, revealed differences among means in both Experiments III and V. These were highly significant ($p < .01$) in Experiment III, based largely upon an increase from control to low dose group, and significant ($p < .05$) in Experiment V, with the significance based largely upon a decrease from low to high dose group mean. However, in both cases there is an increase from control to low dose group followed by a decrease from low to high dose group. The magnitude of the differences are such that this would not appear to be a highly reliable trait in the ascertainment of mutagenic damage in this germ cell/generation test group. Nevertheless, the pattern provides evidence for heritability of the increase from control to low dose group.

Also of interest in respect to heritability are the tail length and fecal rate traits, although significant differences among means are limited to the tail length trait in Experiment V. Of greater importance, the traits tail length and fecal rate in both Experiments III and V show patterns that parallel precisely the body weight trait in Experiments III and V, and all three reinforce a general heritability of the pattern found in the F_1 spermatogonial test results. More specifically, in both experiments, for all three traits, there is an increase from control to low dose group that is significant always in the F_1 spermatogonial test results but only half the time in the F_2 spermatogonial test results. The magnitude of the difference between control and low dose group means is uniformally lesser in the F_2 spermatogonial results than in F_1 spermatogonial results, as might be expected if the genetic variance were diluted as by natural selection in the passage from one to the next generation.

* * *

As a final note it is emphasized that we have concentrated in this report upon those traits in which highly significant differences among means were found in at least one test and where there was support of direction of change of means in other tests. For obvious reasons, consistency of change of direction as a mutagenic effect is important in the development of confidence in this approach. However, the pioneering nature of this effort is emphasized. When one is dealing with the numbers of organisms dealt with in this study, there is a high level of confidence that those differences between dose group means that are highly significant really do reflect real differences due at least in part to mutagenic effects induced. It is perfectly reasonable to suppose that these differences, based as they are upon different mutagenic events are real effects. Of course unnoticed environmental differences between replicates, even in our carefully controlled animal colony rooms, could lead to different effects based upon interaction of genotype and environment.

The nature of the statistical analyses employed (factorial analysis of variance considering the effects of variation in mutagenic dose, sex of organism and in temporally spaced replicates, in addition to a partitioning out of the effects of litter size by covariance) is a highly sophisticated tool. The use of traits that are known to vary easily with environmental effects as, for example, body weight, has been criticized. However, it is clear that the critics do not appreciate that the fundamental

nature of the analysis of variance technique is to compare variation within dose/sex/replicate group with that variation found to exist between groups. Our animals have been carefully coded so that the investigators and workers are not aware of the dose group to which a given individual belongs. Further, all dose groups are distributed randomly within the same colony rooms. They receive the same food, the same water and are handled by the same assistants. Clearly environmental factors may vary in time, and this is very likely a major source of the variation that has been recorded in this study between replicates. But environmental factors that are known to affect a given trait are absolutely the same, insofar as they can be controlled, for all animals within an experimental replicate. If our statistical analysis says the trait is a good one, it is a good one irrespective of philosophical opinion to the contrary. The proof of the pudding, in such case, clearly lies in the eating thereof.

We have emphasized the practical approach in these studies. It is felt that justification has been provided for the investigative employment and for further theoretical exploration of this general approach. These most promising tests would be based upon the traits outlined above in the F_1 generation of spermatogonial treatment and of spermatozoan treatment, with the greater reliability at this time resting with the former. At present, this approach appears to be more useful in the detection of very slight mutagenic damage than of great mutagenic damage, but further investigation, perhaps involving additional traits, may well reveal some that will be affected only by higher mutagenic doses. There were indications that some other inbred strains, for example C3H/HeJ, would bear further investigation. Although considerably less productive than the strain reported upon here, there were evidences that mutagenic events might be more strongly reflected for some traits in this strain than in the strain receiving primary attention.

Respectfully Submitted

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